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13. ABSTRACT *(Maximum 200 words)*

Polymeric materials, particularly fiber reinforced composites (FRPCs) are strategically important to the Air Force. We have shown that common fungi form biofilms on five types of FRPCs. Our data indicate that the fungi penetrate the material. They utilize the resins and sizing chemicals as energy and carbon sources. We used electrochemical impedence spectroscopy (EIS) to investigate degradation of the FRPCs. We demonstrated a progressive decline in impedence in 179 days as a result of fungal growth on the FRPCs. Our data indicate that the composite materials are susceptible to microbial attack in moist conditions at ambient temperatures.

We also investigated the effects of chromium, used in surface coatings, on microbial activity. Initial tests showed that both hexavalent and trivalent chromium inhibits bacterial activity. However, the bacteria rapidly develop resistance to chromium.

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FINAL REPORT

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Grant Number	F49620-95-1-0151
Principal Investigator	Professor Ralph Mitchell Division of Engineering and Applied Sciences Harvard University Cambridge, MA 02138

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1. ABSTRACT

Polymeric materials including electronic insulation polyimides and fiber-reinforced polymeric composites (FRPCs) are strategically important materials to the Air Force. Stability of these materials is vital to their proper function. A mixed culture of fungi, enriched from degraded polymeric materials, was capable of forming biofilms on coupons of five types of FRPCs. They also grew actively in aqueous extracts of these composites under ambient conditions. The data indicated that the fungi utilized the resins or fiber chemical sizing as carbon and energy sources. Mechanisms of composite degradation were monitored using electrochemical impedance spectroscopy (EIS). For example, a progressive decline in impedance from above 10^7 Ohms to below 10^6 Ohms was detected in the inoculated panels of epoxy resin/carbon fibers composite by EIS after 179 days of incubation, but not on the sterile controls. The degradation proceeds through an initial ingress of water into the resins, followed by degradation of bonding between fiber surfaces and resins and finally separation of fibers from the resins. At the end of EIS study, the extent of disbonding in the inoculated composite was greater than the control, observed by a three-point bending test and scanning electron microscopy. These results suggested that the composite materials are susceptible to microbial attack by providing nutrients for growth.

The most common primer used on U.S. Air Force aircraft surface coatings contains significant quantities of chromium (Cr). Coating waste poses an environmental threat, because of the presence of Cr. Inhibition of microbial growth by hexavalent (Cr, VI) and trivalent (Cr, III) was investigated using both a pure culture of *Pseudomonas aeruginosa* and a mixed culture of a natural population. Our data indicate that the critical concentrations for inhibition to be observed were >0.50 mM for Cr(III) and >0.20 mM for Cr(VI) by optical density (OD) measurements, and >0.10 mM for Cr(III) and 0.50 mM for Cr(VI) by dehydrogenase assay. Development of biocidal activity against microorganisms

was observed in 24 hours by OD determination and 48 hours by dehydrogenase measurement. However, the initial inhibition by Cr can be overcome during extensive contact, because microorganisms develop resistance. No apparent difference in colony-forming units (CFU's) of *Pseudomonas aeruginosa* between various concentrations of Cr (VI) (0 - 0.30 mM) and Cr(III) (0 - 0.5 mM) was detected after 48 hours. Similar results were observed in a mixed culture of a natural population of bacteria with the same treatment. These results indicate that bacteria are capable of resisting Cr. Production of exopolysaccharide which complexes chromium and acquisition of plasmids may mediate resistance to Cr.

Key Words: Biodeterioration; Degradation; Disbonding; Electrochemical impedance spectroscopy; Fungi; Polymeric composites

2. OBJECTIVES

This research program had two objectives. The first was to investigate the biodegradation of fiber reinforced polymeric composites (FRPC's) and their constituents. A second goal of this research was to determine the mechanisms involved during biodegradation of protective polymeric coatings, specifically the role of Cr in protection against biofilm formation. Hexavalent chromium (Cr) is a constituent of primers of a number of coatings of interest to the Air Force. The importance of chromium as a biocide in these coatings and its protective action against microbial activity was investigated in our study.

3. EXPERIMENTAL METHODS

3.1 Colonization of composite coupons

The FRPC materials were Magnamite IM-6G/3501-6 unidirectional layup with F161/120 style glass prepreg on both sides (Hercules Inc., Magna, UT). They consisted of Hercules 3501-6 epoxy resins with unidirectional reinforced graphite fibers with a layer of crowfoot satin weave epoxy impregnated glass on both sides. The thickness of the composite was approximately 2 mm. Fiber volume was 62% in the composite. The graphite fibers and epoxy resins were cured into flat plates for use as assembly racks. Large pieces were cut into small coupons with dimensions of 10 x 20 x 2 mm. The coupons were sterilized and inoculated with our previously isolated fungal consortium in flasks containing a malt broth medium (Difco Lab., Detroit, Michigan). The consortium was previously enriched on degraded polymeric materials. After 30 days of incubation, samples were prepared for scanning electron microscopic observation.

3.2 Growth on composite extracts

In this experiment, two coupons of each composite type were autoclaved in an individual flask containing 80 mL of a minimum salt medium for 20 min. The salt medium consisted of (g L^{-1}): K_2HPO_4 0.8, KH_2PO_4 0.2, $CaSO_4 \bullet 2H_2O$ 0.05, $MgSO_4 \bullet 7H_2O$ 0.5, $FeSO_4 \bullet 7H_2O$ 0.01, and $(NH_4)_2SO_4$ 1.0. The coupons were autoclaved once in the medium to extract soluble organics and then removed. The composite extracts were inoculated with 100 μL of the fungal consortium. Culture aliquots for growth were measured spectrophotometrically at 600 nm. Triplicate samples were used in the experiment.

3.3 Sample preparation for scanning electron microscopy (SEM)

Polymeric composite samples from exposure tests or the inoculated and sterile EIS cells were treated with 3% glutaraldehyde buffered with 0.2 *M* sodium cacodylate

overnight. The solution was previously filtered through a 0.2-μm-pore-size polycarbonate membrane filter (Gelman Science, Ann Arbor, MI). Samples were washed with 0.2 *M* Na cacodylate three times, fixed in 1% osmium tetroxide with 0.1 *M* Na cacodylate, and rinsed with 0.2 *M* Na cacodylate and deionized water three times for each treatment. The samples were dehydrated by immersing in an ethanol-distilled water series of 40, 60 70 and 80 %, and 85, 90, 95 and 100% ethanol. Samples were stored in 100% ethanol and air-tight sealed glass vials before being critical point dried in liquid CO_2 (Smdri PVT-3B, Tousimis Research Co., Rockville, MD). Following drying, they were immediately coated with gold-palladium and viewed under an AMR 1000 scanning electron microscope.

3.4 Electrochemical impedance spectroscopy (EIS)

Coupons of the composites were prepared for EIS monitoring. EIS cells were constructed by gluing a piece of FRPC onto a 316 stainless steel coupon (50.0 X 50.0 mm) with a conductive silver epoxy (SPI Instrumental, West Chester, Pennsylvania). A schematic illustration of the EIS cells is shown in Figure 1. On the composite surface, a 30.0 mm long acrylic tube (I.D., 34.9 mm; O.D. 38.1 mm) was attached to the polymer-stainless steel coupon by Epon 828 epoxy resin (Shell Chemical Co., Houston, Texas) cured with Amercoat 90 resin (Ameron, Protective Coatings Group, Brea, California) in a ratio of 1:4. After curing the adhesive, the internal surfaces of the tube and the composite directly exposed to the internal of the tube were thoroughly sterilized with 70% ethanol and dried in a laminar-flow sterile hood. Initially, a volume of 15.0 mL of sterile 0.2 *M* NaCl solution and 1.0 mL of the salt medium (described above) was added to the acrylic tube of the EIS cell as a working electrode, and measurement of the impedance responses was made after equilibration of the system. The uniformity of all prepared EIS cells was evaluated by EIS spectra analyzed in magnitude, phase angle and Nyquist complex plane plots to determine the validity of using them in subsequent spectroscopy monitoring. Then, a set (three) of the prepared EIS cells were inoculated with 100 μL of the fungal

consortium maintained on the malt extract medium. Another set (three) of EIS cells was kept sterile throughout the study as control. Aseptic procedures were used throughout the EIS measurements to avoid contamination and cross contamination of the EIS cells.

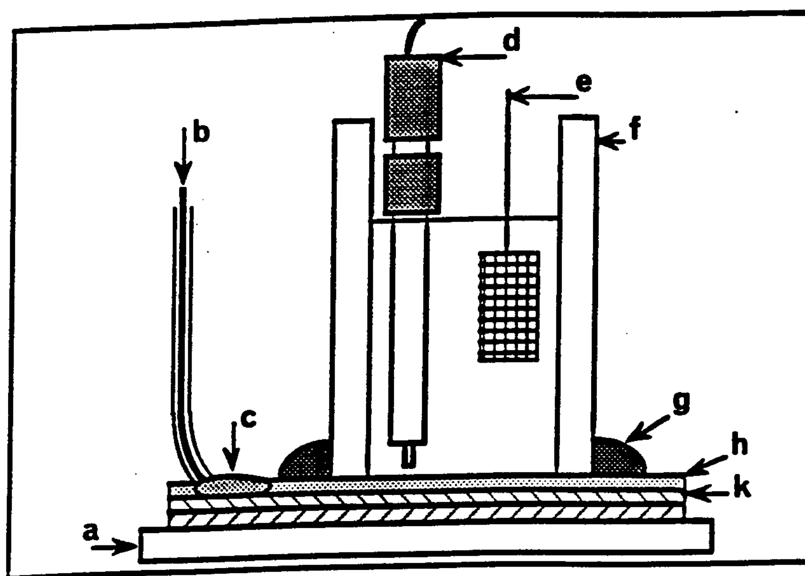


Figure 1. Schematic illustration of an EIS cell used in electrochemical impedance study of composite degradation. *a*, Plexiglass base; *b*, working electrode connection; *c*, silver loaded epoxy; *d*, standard calomel electrode; *e*, platinum counter electrode; *f*, Pexiglass cell to hold NaCl electrolyte solution; *g*, Amercoat; *h*, composite coupon; and *k*, mettalic substratum.

Our EIS system consists of a Schlumberger 1250 frequency response analyzer combined with a Schlumberger 1286 electrochemical interface (Schlumberger Technologies - Instruments Division, Billerica, Massachusetts). *Z*-plot software (Scribner Associates, Inc., Charlottesville, Virginia) was used to manipulate the system. During data acquisition, samples were potentiostatically held at their open circuit potential (OCP), and a sinusoidal perturbation of 20-50 mV was applied to the system. Impedance responses were measured over a range of frequencies from 65 kHz to 1 mHz and spectra were recorded as a function of immersion time at ambient temperature and pressure. OCPs were monitored versus a saturated calomel electrode as a reference electrode of the tri-electrode system. Platinum mesh was used in the EIS cell as a counter electrode, and the constructed EIS cell as a working electrode. Both Bode magnitude and phase angle plots as well as the Nyquist complex plane plots were used to provide information on increases in porosity, local defects and disbonding.

3.5 Interlaminar Shear Strength Test

At the end of our EIS monitoring tests, composites from the inoculated and control EIS cells were sectioned with a diamond-tipped wet saw blade to an approximately dimension of 38 mm$\times$1.3 mm for mechanical analysis. Specimens were tested in a three-point flexure with a span-to-depth ratio of 6:1 to promote interlaminar shear failure. Thin rubber pads were placed between the contact pins and the specimen to prevent premature surface damage at these locations. Failure modes were noted and interlaminar shear properties were observed with SEM.

3.6 Microbiological assays

Pure culture of *Pseudomonas aeruginosa* 6135 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The lyophilized dry cells in an ampule were aseptically transferred into culture flasks containing 100 mL of sterile nutrient broth

(Difco Lab., Detroit, MI). After 24 hours of incubation at 30°C, the culture in the flask was centrifuged into pellets and the supernatants discarded. These pellets were washed once with 50 mL of a sterile phosphate buffer, containing K_2HPO_4, 5.2g/L, KH_2PO_4, 5.3g/L, and then diluted to desirable concentrations.

Concentrations of Cr (VI) and Cr(III) used were 0, 0.05, 0.10, 0.25 and 0.50 mM for *P. aeruginosa*, and 0, 0.05, 0.10, 0.20, 0.30 mM for mixed culture studies. Initial concentrations of bacteria were 103 cells/L in both experiments. Following incubation, bacterial survival or growth was monitored by two different techniques, optical density (OD) spectrophotometrically at 600 nm to estimate the numbers of bacterial cells, and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) for dehydrogenase activity of metabolically active bacteria.

4. RESULTS

4.1 Biological deterioration of composites

Our results showed that all five composite materials being studied were susceptible to attack by a mixed culture of fungi, enriched from degraded composite materials. SEM observation indicated fungal colonization of the composite surfaces and localized penetration of fungal hyphae into the interior of the composite resins, particularly for the material containing a fluorinated polyimide resin reinforced with glass fibers (Figure 2). The microorganisms were capable of growing on sizing chemicals on surfaces of fibers (Figure 3).

Figure 2. Scanning electron micrograph showing colonization and penetration of a fluorinated polyimide resin/carbon fiber composite by hyphae of fingi (×2000)

Figure 3. Scanning electron micrograph of a natural population of bacteria growing on the surface of a carbon fiber utilizing sizing chemical as carbon and energy sources (×1000)

In a further investigation, inoculation of aqueous extracts of whole composite coupons with our fungal inoculum resulted in significant stimulation of fungal growth (Figure 4). These data support the hypothesis that organic carbon components of the composite materials provide essential nutrients for microbial growth. This is the first direct indication of the nutritional relationship between the FRPC deterioration and the growth of fungi. The data also confirm observations from an earlier study that composite fibers serve as carbon and energy sources for the growth of microorganisms. Composites contain a range of chemicals to improve the material properties, including plasticizers, flame retardants, catalysts, and colorants. Most of these additives are biodegradable, particularly polyesters under both aerobic and anaerobic conditions.

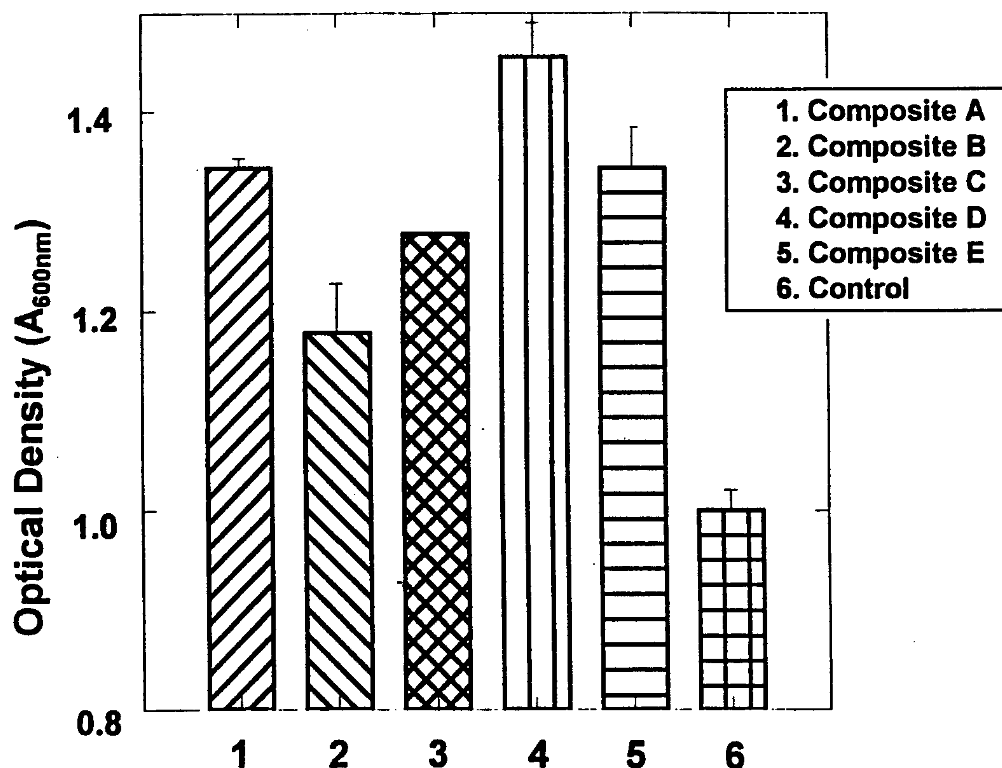


Figure 4. Microbial growth on extracts of composites. Coupons of composites were extracted in a minimum salt medium by autoclaving. Our fungal culture was inoculated into the extract and growth of fungi was monitored spectrophotometrically over time.

The success or failure of an FRPC is also governed by the degree of adhesion between the fiber surface and the resin matrix. Electrochemical impedance spectroscopy (EIS) has been used for assessment of failure of protective coating, electronic packaging polyimides, and composites. Localized weakening of the bonding between the reinforcing fibers and the resin matrix will result in fiber disbonding and delamination when the material is under stress. When this happens, a decrease of impedance is detected. To our knowledge, no investigation of microbial degradation of composites has been reported using EIS.

In our study, EIS spectra of composite coupons with fungal biofilms showed large deviations from the initial spectra. A decrease of impedance was observed in the high frequency region with a bending point between 10^3 and 10^4 Hz within 179 days (Figure 5a). A phase angle plot showed correspondingly continuous bending away from the initial spectrum (Figure 5b), indicating a decrease of pore resistance of the composite matrix or an increase of pore size and pore numbers. The development and compression of the semicircles, indicated by curves bending away from those at the begining of experiment in the Nyquist complex plane plots (Figure 5c), also confirmed that the conductance of the composite increased over time of incubation as composite matrix deteriorates progressively.

During the 179 days of monitoring, EIS spectra in the magnitude, Bode and Nyquist complex plane plots collectively demonstrated that there was a continuous deterioration of the composite matrix after inoculation. However, the composite maintained under sterile conditions showed minimal changes of the impedance and phase angle (Figures 6a and 6b), and almost no change in the Nyquist complex plane plot (Figure 6c). The small deviation between measurements at different samplings can be explained by the variation in electrical noise and water permeation into the resin. The magnitude of

impedance showed that the composite was intact when maintained under sterile conditions after 179 days.

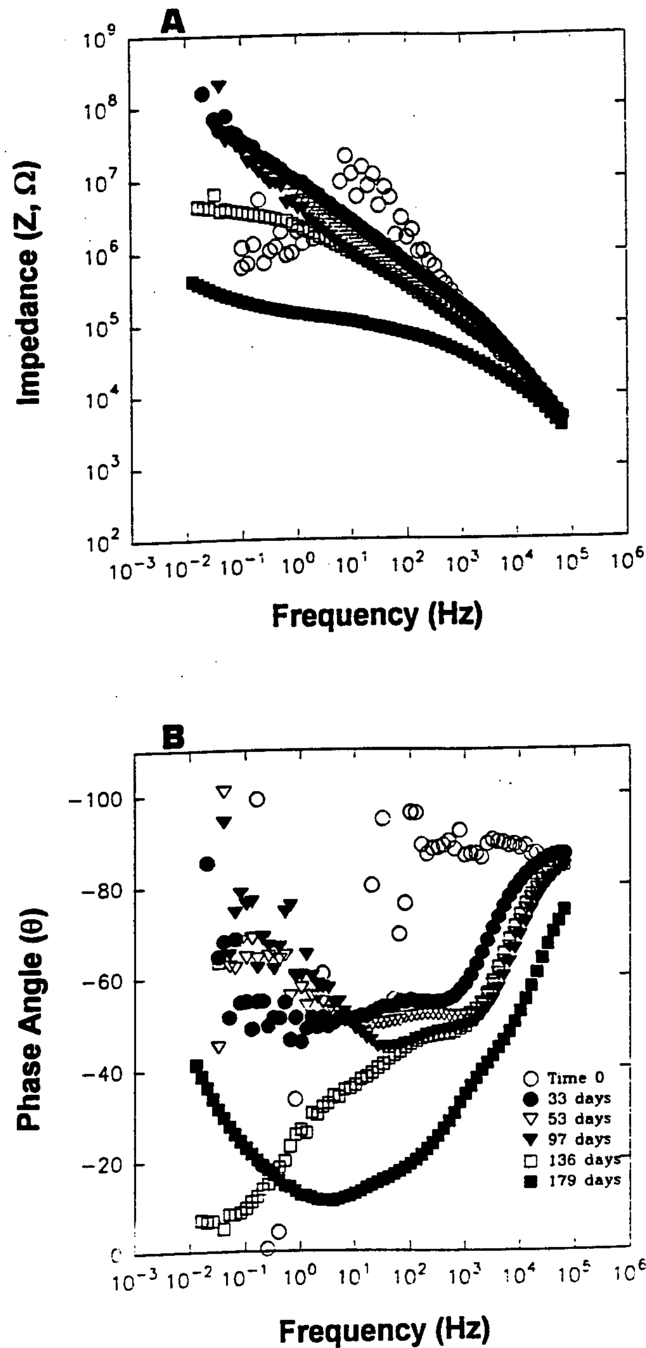


Figure 5. EIS spectra of epoxy resin/carbon fibers composite showing changes in (a) Bode magnitude, (b) phase angle and (c) Nyquist plots after inoculation with fungal culture

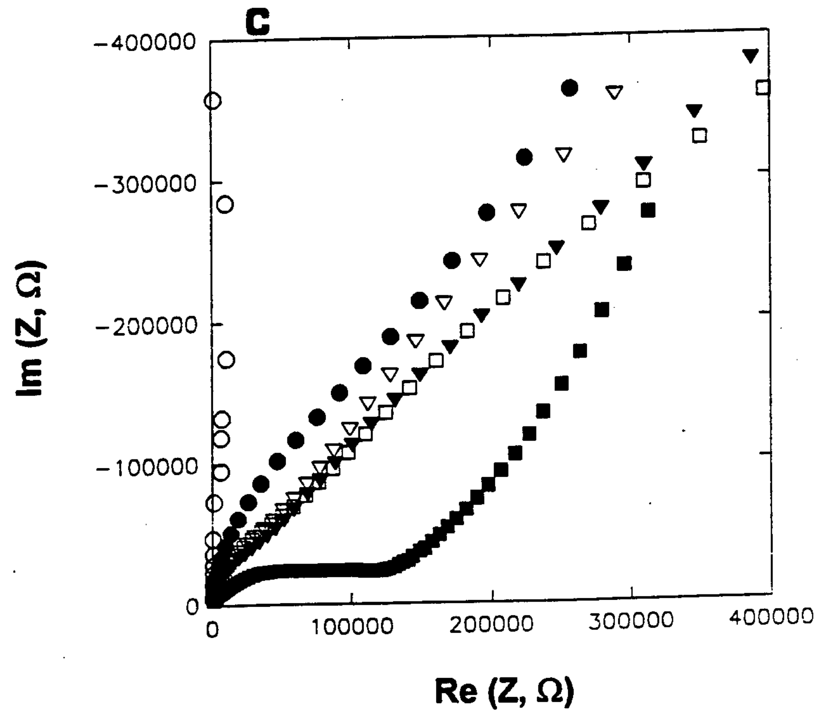


Figure 5. *(Continued)*

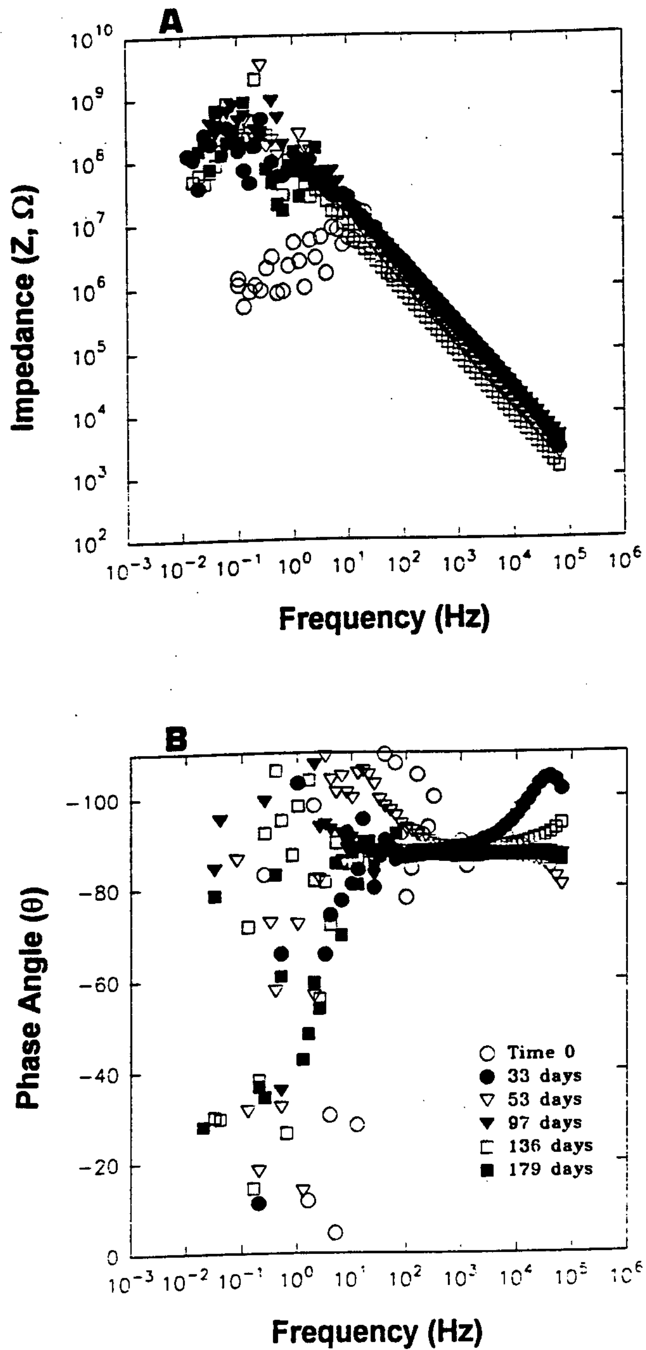


Figure 6. EIS spectra of epoxy resin/carbon fibers composite showing changes in (a) Bode magnitude, (b) phase angle and (c) Nyquist under sterile condition

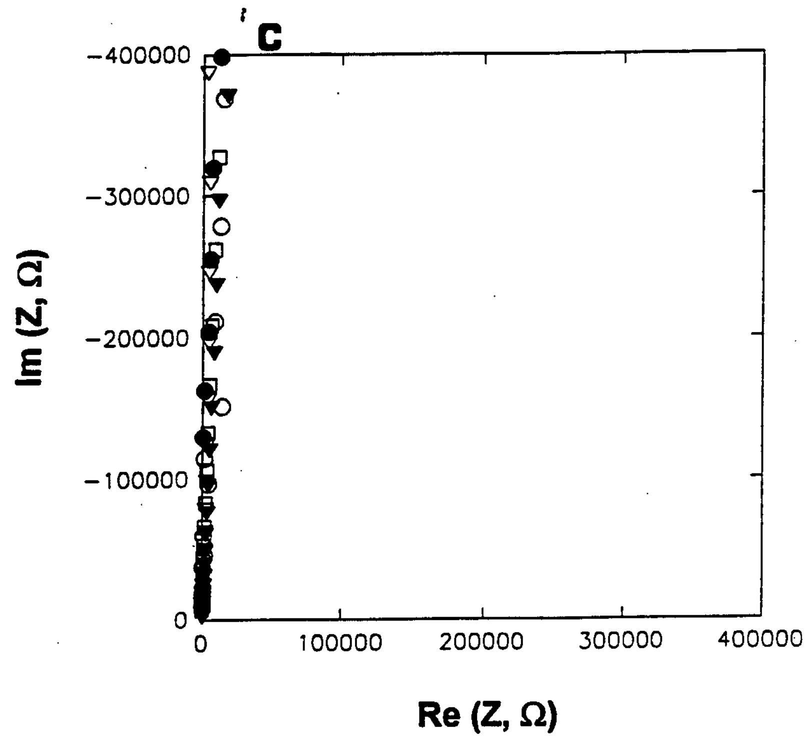


Figure 6. *(Continued)*

Composites from the inoculated EIS cells developed fungal hyphae and spores on surfaces of the composite, while no microorganisms were observed on surfaces of the control at the end of EIS monitoring. Mechanical analysis was performed on the composites exposed to fungi and those kept in sterile conditions. No significant difference of interlaminar shear strength between the inoculated and the control composites was detected. However, the inoculated samples fractured. The fracture indicated that bonding strength between fibers and resins was weakened after inoculation with the fungi (Figure 7). The inability of the mechanical test to detect any differences between inoculated and control may be due to the insensitivity of the technique to the small proportion of disbonding over the whole composite matrix.

Since sizing chemicals, including starch derivatives, acetylated celluloses, and vinyl esters are biodegradable, delamination of fibers from resin matrices is likely to occur as a result of their microbial decomposition. These failures may be avoided by using a sizing that is resistant to microbial attack or by incorporating a biocide. Organics in resins which may support growth of microorganisms can be either eliminated or inactivated by incorporation of a biocide in the matrix. Elimination of microbial contamination in the composite manufacturing process would provide additional protection against biological deterioration.

4.2 Biotoxicity assay of Cr to microorganisms

Another objective of this study was to evaluate the performance of polymeric coatings of interest to the Air Force during exposure to microorganisms. Chromate is a significant constituent of primer coatings. The role of Cr as a biocide has been examined by culturing *Pseudomonas aeruginosa*, a common bacterial species, with varying concentrations of $K_2Cr_2O_7$ [Cr(VI)] and $CrCl_3$ [Cr(III)]. Bacterial survival or growth was monitored by two different techniques, optical density (OD) to estimate the numbers

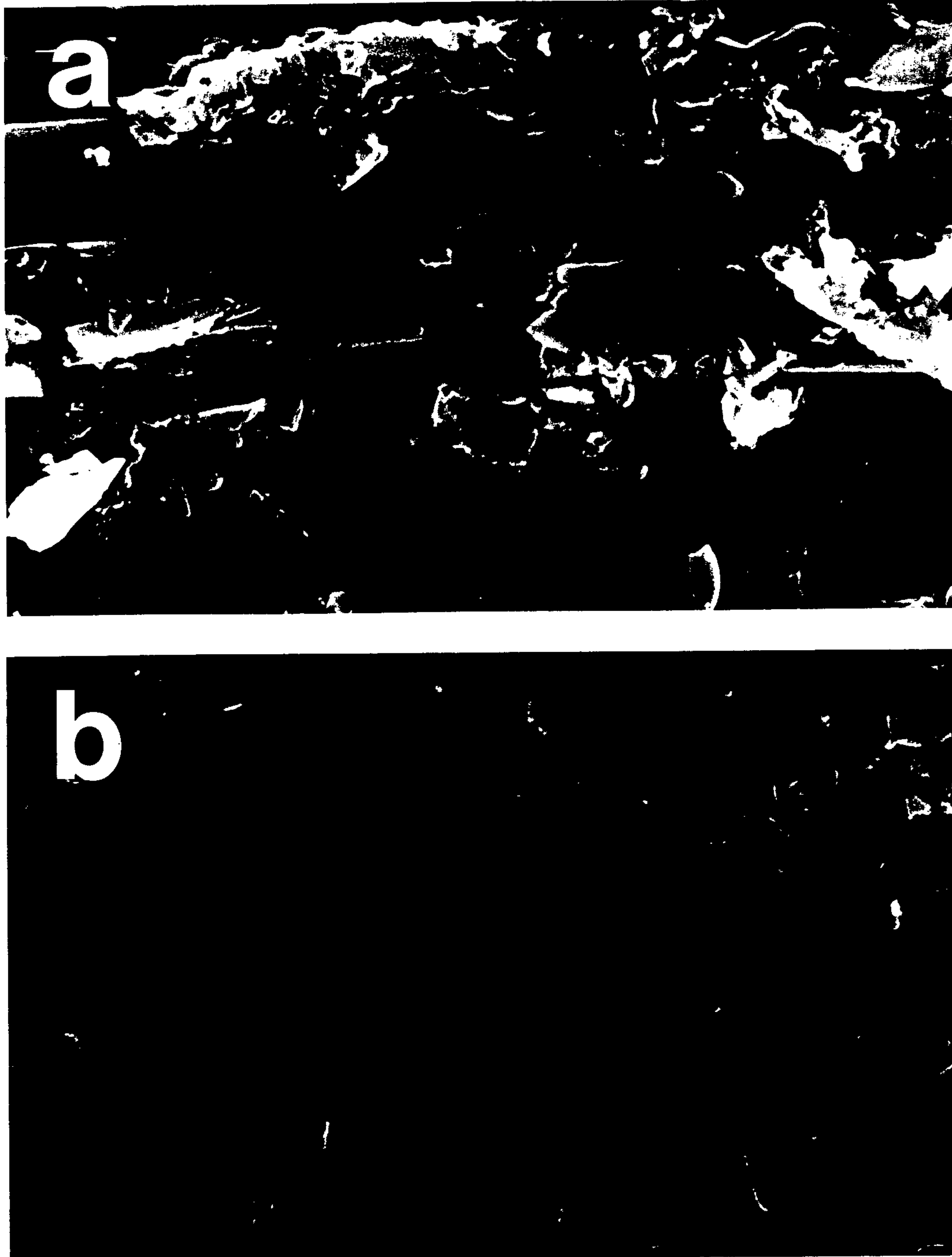
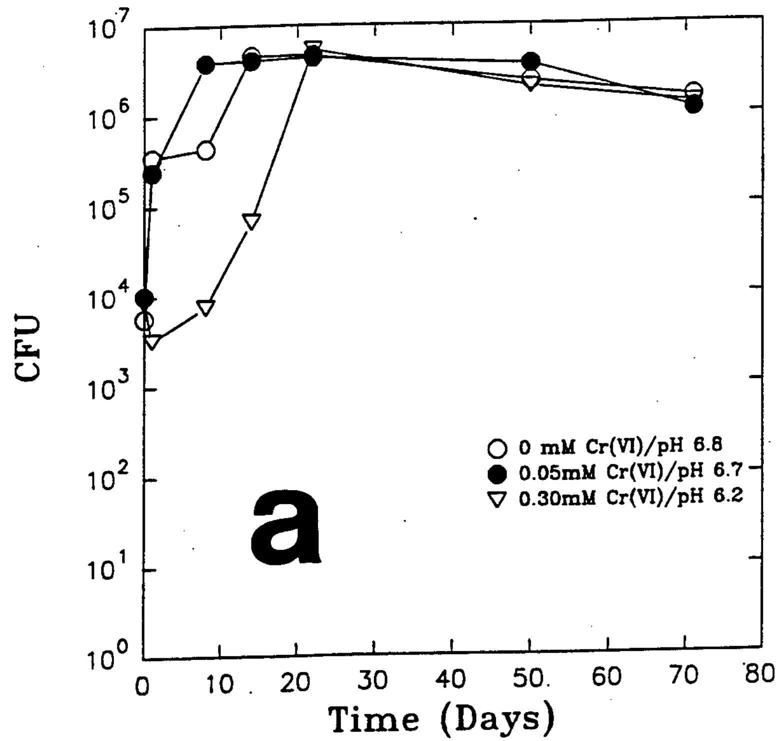


Figure 7. Scanning electron micrographs of fracture morphology showing disbonding between epoxy resin and carbon fiber after (*a*) inoculation with fungi (×2000), and (*b*) kept under sterile conditions (×1000) for 179 days

of bacterial cells, and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) for dehydrogenase activity. Our preliminary data indicate that the critical concentrations for toxicity to be observed were >0.50 mM for Cr(III) and >0.20 mM for Cr(VI) by OD measurements, and >0.10 mM for Cr(III) and 0.50 mM for Cr(VI) by dehydrogenase assay. Development of toxicity for bacteria was shown in 24 hours by OD determination and 48 hours by dehydrogenase measurement.

The initial toxicity from Cr was overcome during extensive incubation of the bacteria. No apparent difference in colony-forming units (CFU's) of *Pseudomonas aeruginosa* between various concentrations of Cr (VI) (0 - 0.30 mM) and Cr(III) (0 - 0.5 mM) was detected after 48 hours (Figure 8). Similar results were observed on a mixed culture of a natural population of bacteria with the same treatment. These data suggest that bacteria are capable of either detoxifying chromium by transformation to nontoxic species or development of other mechanisms. Possible mechanisms involved may be either the production of exopolysaccharides which complex chromium and/or plasmid mediated resistance.



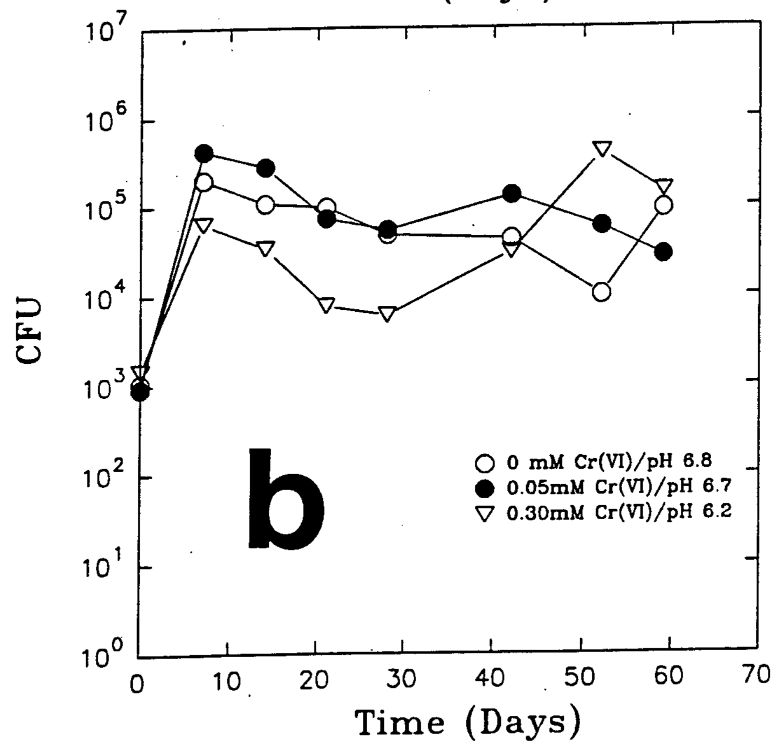


Figure 8. Inhibitory effects of Cr (VI) on microbial growth at various concentrations. (a) *Pseudomonas aeruginosa*; (b) a mixed culture of a natural population

5. CONCLUSIONS

Composite constituents such as matrix resins, additives and fibers are susceptible to microbial growth, resulting in potential damage to the resins and delamination of the fibers from resin matrices. Microorganisms may obtain energy from chemicals in resins and on the fiber surfaces. Fungi were shown to be responsible for the degradation of composite materials, and it appeared that sizing chemicals and resin constituents were readily degraded by fungi.

Chromium in haxavalent and trivalent states inhibits growth of pure and mixed cultures of microorganisms. However, the microorganisms are capable of overcoming the inhibitory effect within a relatively short period of time, approximately 48 hours in our experiments.

6. PERSONNEL SUPPORTED

Dr. Ji-Dong Gu

7. PUBLICATIONS AND PRESENTATIONS

Gu, J.-D., C. Lu, T.E. Ford, K.E.G. Thorp, and R. Mitchell. 1996. Fungal degradation of fiber-reinforced composite materials. *CORROSION/96*, Paper No. 275, NACE International, Houston, TX.

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