

REPORT DOCUMENTATION PAGE

(2)

1a AD-A245 511			1b RESTRICTIVE MARKINGS N/A		
2a 			3 DISTRIBUTION AVAILABILITY OF REPORT		
2b /A			Distribution Unlimited		
4 PERFORMING ORGANIZATION REPORT NUMBER(S) University of Hawaii			5 MONITORING ORGANIZATION REPORT NUMBER N/A		
6a NAME OF PERFORMING ORGANIZATION University of Hawaii		6b OFFICE SYMBOL (If applicable) N/A		7a NAME OF MONITORING ORGANIZATION Office of Naval Research	
6c ADDRESS (City, State, and ZIP Code) Hawaii Institute of Geophysics 1000 Pope Road, Honolulu, HI 96822		7b ADDRESS (City, State, and ZIP Code) 800 N. Quincy Street Arlington, VA 22217-5000			
8a NAME OF FUNDING/SPONSORING ORGANIZATION Office of Naval Research		8b OFFICE SYMBOL (If applicable) ONR		9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER N00014-88-K-0044	
8c ADDRESS (City, State, and ZIP Code) 800 N. Quincy Street Arlington, VA 22217-5000		10 SOURCE OF FUNDING NUMBERS			
		PROGRAM ELEMENT NO 61153N		PROJECT NO RR04106	
				TASK NO 44/X-XXX	
				WORK UNIT ACCESSION NO	
11 TITLE (Include Security Classification) (U) Examination of Chemical Adsorption and Marine Biofouling on Metal Surfaces using Raman Scattering Techniques and Electrochemical Impedance Spectroscopy					
12 PERSONAL AUTHOR(S) Taylor, G.T., Sharma, S.K., Liebert, B.E., Mower, H.F.					
13a TYPE OF REPORT FINAL		13b TIME COVERED FROM 11/87 TO 12/90		14 DATE OF REPORT (Year, Month, Day) 1991, March 14	
15 PAGE COUNT 6					
16 SUPPLEMENTARY NOTATION					
17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP	Biofouling, Chemical Adsorption, Raman Spectroscopy, Infrared Spectroscopy, Electrochemical Impedance Spectroscopy		
06	03				
19. ABSTRACT (Continue on reverse if necessary and identify by block number)					
A suite of spectroscopic, optical, and electrochemical techniques to examine initial stages of biofilm formation were developed and evaluated. Using a combination of ellipsometry, FT-IR reflectance-absorbance spectroscopy, and electrochemical impedance spectroscopy, we were able to gain quantitative, compositional, and structural information about the substratum-adsorbate interface for the model protein, Ribulose 1,5-Bisphosphate Carboxylase-Oxygenase. Adsorption isotherms for RuBisCO on titanium, copper, iron, glass, polycarbonate, and teflon were obtained. Spectroscopic evidence suggests that molecular structure and electrical impedance vary with protein surface coverage. Furthermore, bioavailability of sorbed protein and degradation rates appear to vary with coverage resulting from altered structural conformation.					
(over)					
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS			21 ABSTRACT SECURITY CLASSIFICATION (U)		
22a NAME OF RESPONSIBLE INDIVIDUAL M. Marron			22b TELEPHONE (Include Area Code) (703) 696-4760		22c OFFICE SYMBOL ONR

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SECURITY CLASSIFICATION OF THIS PAGE

92 1 22 014

92-01780



19. (cont'd.)

Field deployments of 70 test surfaces comprised of ten materials in oceanic waters were performed for 3 days in June and July 1990. Bio-films were dominated by bacteria; diatoms and protists were rare. Bacterial colonies were rare on surfaces, so data is characteristic of initial settlement. Surfaces with highest and lowest critical surface free energy had the highest settlement rates and surfaces with 20-30 mN/m surface free energies had the least. Even toxic metals, such as copper and copper nickel alloys, had significant settlement, but smaller cell size and lower population diversity. Further studies on isolates from these materials are underway.



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FINAL REPORT

CONTRACT#: N00014-88-K-0044

R&T CODE: 441h005

PRINCIPAL INVESTIGATOR: Gordon T. Taylor*

CO-INVESTIGATORS: Shiv K. Sharma, Bruce E. Liebert & Howard F. Mower

INSTITUTE: University of Hawaii at Manoa

CONTRACT TITLE: Examination of Chemical Adsorption and Marine Biofouling on Metal Surfaces Using Raman Scattering Techniques and Electrochemical Impedance Spectroscopy

PERIOD OF PERFORMANCE: 1 November 1987 - 31 December 1990

OBJECTIVES: Our primary objective was to investigate the initial chemical and microbiological processes which occur on virgin metal and polymer surfaces immersed in seawater using primarily non-invasive, analytical techniques, and to ascertain how these processes are governed by bulk seawater composition, substratum surface energy and reactivity, flow regime, and microbial population dynamics. To accomplish this, we defined the following specific objectives.

- (1) Develop optimal spectroscopic sampling strategies for detection, identification, and quantification of low concentrations of adsorbed organic materials, including FT-IR spectrophotometry, Raman Scattering spectroscopy, Electrochemical Impedance spectroscopy (EIS), and ellipsometry.
- (2) Intercalibrate spectroscopic techniques with radiometric and biochemical analyses of labeled model compounds, such as the protein, RuBisCO (Ribulose Bisphosphate Carboxylase-Oxygenase).
- (3) Examine adsorption of surfactants, such as proteins, on substrata as functions of bulk concentration, critical surface energy of substratum, and exposure time of substratum to seawater.
- (4) In the field, examine primary chemical adsorption and microbial attachment to a variety of surfaces (metals, glasses, and polymers) exposed to pelagic waters for short durations (≤ 3 days).
- (5) In the laboratory, using continuous culture techniques, quantify rates of microbial attachment to a variety of substrata (metals, glasses, and polymers) under varying biomass, and flow regimes.

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ACCOMPLISHMENTS: To examine the formation of natural, organic, thin films on materials in seawater, we have adapted several non-destructive techniques which yield quantitative and qualitative information on the film and underlying substratum. We developed a Reflection-Absorption accessory for our BOMEM DA3 FT-IR/Raman spectrophotometer, which permits spectroscopic examination of thin films deposited on many optically flat, reflective substrata. Film thicknesses were determined by ellipsometry which permits measurement of both adsorbed film thickness and underlying metal oxide layer. The oxidation potential as a function of film thickness was examined using Electrochemical Impedance Spectroscopic (EIS) over a frequency range of 100 μ Hz to 100 kHz. Actual surface coverages were determined radiometrically using tritiated RuBisCO. Although natural thin films were detectable using Raman scattering techniques, satisfactory progress was impeded by fluorescence and sensitivity problems, so the more tractable FT-IR approach was pursued.

Using this combination of optical, electrochemical, and radiometric techniques and the protein RuBisCO as a model adsorbate on titanium, copper, and iron, we have been able to determine: (i) organic film thicknesses down to 0.5 nm (ellipsometry), (ii) underlying oxide layer thicknesses (ellipsometry), (iii) chemical class of adsorbate by functional group identification on films as thin as 4 nm (FT-IR), (iv) surface coverage (FT-IR, ellipsometry, radiometry), (v) adsorbate structural change as function of surface concentration (FT-IR), and (vi) corrosion potential (EIS). Using an adsorption isotherm approach, we have found excellent sensitivity and agreement between these techniques over a wide bulk concentration range. We have also observed marked differences between metals in their ability to bind protein. For example, copper has a much higher affinity and binding capacity for RuBisCO in seawater than does titanium. Therefore, under the same conditions, copper will scavenge more protein from solution and much thicker organic films will form on its surface than on titanium (as much as 70 times more). Although not as amenable to optical techniques because of optical absorptive properties, we have also derived adsorption isotherms of RuBisCO on TFE Teflon, Pyrex glass, iron, and polycarbonate.

In our studies of RuBisCO adsorption to Ti at low surface coverages (≤ 1 molecular layer), IR vibrational frequencies for Amide I and II bands were observed to shift significantly with decreasing coverage. Above monomolecular coverages, Amide frequencies were relatively constant. The behavior of Amide frequencies suggests the existence of unique protein conformations dependent upon available adsorption sites and molecular crowding. Observed frequency shifts and the quantitative relationship of Amide I and II bands are similar to published observations of denatured mammalian proteins, suggesting changes in tertiary as well as secondary structure, i.e., relative abundances of α -helix, β -sheet, and random conformations. FT-IR analysis of aqueous suspensions of denatured RuBisCO and RuBisCO renatured in an α -helix promoter, trifluoroethanol, were performed to aid in interpretation of adsorption isotherm data. Furthermore, electrochemical impedance across the interface varies in two phases with surface coverage; being most positively dependent in the monomolecular region and less dependent in the multimolecular region. Thick protein layers appear to be effective insulators to electron flow across the interface.

To examine the biological/biogeochemical significance of protein conformational states, bacterial degradation studies were conducted using ^3H -RuBisCO adsorbed to glass beads in relatively thin and thick films. Parallel degradation isotherms for aqueous ^3H -RuBisCO were also obtained. ^3H -labeled pools were monitored to examine desorption, proteolysis, bacterial assimilation, and respiration as functions of surface coverage and bulk phase concentration. Experimental results are currently under analysis.

A field array consisting of anchor, floats, and Delrin support which holds 70 coupons for spectroscopic, biochemical, microbiological, epifluorescence microscopy, and SEM analyses and 5 electrodes for EIS was deployed for 3 days at 10 m depth in relatively pelagic waters 2 km off the south shore of Oahu, Hawaii in June and in July 1990. A variety of surfaces (titanium, iron, stainless steel, copper, copper-nickel alloy, aluminum, Pyrex, quartz glass, Teflon, and Delrin), all with mirror finishes, were exposed to examine the effects of critical surface energy, electrostatic charge, solubility, and toxicity on the primary biofouling events. Microscopic observations indicate that the microbial biofouling "community" of each surface was dominated by bacteria and was unique with respect to surface coverage, cell morphology, and size frequency distribution of the populations. Microbial surface coverage varied as a parabolic function of critical surface energy, high at both extremes and lowest in the 20-30 mN m⁻¹ region. Furthermore, microbial settlement trends observed in June were similar to those of July. Culturing and identification of isolates from each of these surfaces are underway. FT-IR/Raman spectroscopic, ellipsometric, and EIS analyses of replicate test coupons were also performed.

In the laboratory, biofilms on glass coupons generated after 2 and 7 days in flowing offshore seawater amended with inorganic nutrients and subjected to a 12:12 light dark cycle were examined using FT-IR. Both 2- and 7-day coupons had 100-120 nm biofilms when removed. These samples yielded complex FT-IR absorption spectra with pronounced Amide I and II bands suggesting a large protein component in the film. The presence of protein was confirmed using a bicinchoninic acid protein assay (PIERCE MicroBCA assay) on detergent extracts of the surface. Protein yields from the glass coupons were compared in five different sonicated extracts; Phosphate Buffered Saline (PBS) and PBS amended with non-ionic (Triton X-100), anionic (SDS), cationic (hexadecyltrimethyl-ammonium chloride, HDTMAC) or zwitterionic (CHAPS) detergents. Highest protein yields were obtained in HDTMAC and CHAPS and no detectable protein was evident in anionic and non-ionic detergents, suggesting that anionic functional groups play a role in adsorption to glass.

In the laboratory, continuous culture studies to examine bacterial adsorption, attachment and colonization to Pyrex, as functions of time, laminar flow regimes (0.5 - 9 cm sec⁻¹) and nutrient supply were performed under constant growth conditions (0.5 div day⁻¹). These studies demonstrated that the proportion of the total population attached to a substratum varies with nutrient supply and provided some estimate of adhesive strength of bacteria. Continuing studies of isolates from the field experiments are underway to characterize the interactions of their cell surfaces with solid substrata.

SIGNIFICANCE: The primary adsorbed organic layer varies in mass and composition depending on the composition of the underlying substratum. This primary film appears to be macromolecular in nature. We have modeled how substratum characteristics determine film formation of a common protein and how the molecular organization of the interface can affect bioavailability of macromolecules. Subsequent microbial colonization may be influenced by both the primary film and the underlying substratum in very specific ways, such as steric interactions of the surface and the microbial cell wall or toxicity of soluble metal oxides. Our field studies have demonstrated that surface determinants influence bacterial settlement for at least 3 days in oceanic waters.

FUTURE PLANS:

1. Competitive adsorption studies using ^3H -RuBisCO, humic acids, and polysaccharides will be conducted as part of a student's Ph.D. dissertation (P. Troy).
2. Identification of field isolates and characterization of cell surface hydrophobicities will continue as part of a student's Ph.D. dissertation (D. Zheng).
3. Using isolates from hydrophobic and hydrophilic substrata, cell sorption will be examined as functions of substratum critical surface tension and type of conditioning layer.
4. Adhesive strength of the field isolates will be examined in further detail.

INVENTIONS: None

PUBLICATIONS AND REPORTS:

- 1987 Taylor, G.T., S.K. Sharma, B.E. Liebert & H.F. Mower. Examination of primary chemical adsorption and microbial attachment to metal surfaces in the marine environment Raman scattering techniques and AC impedance spectroscopy. Presented at Conf. on Marine Biodeterioration, Nov. 18-20, Univ. Southern Calif., L.A., CA.
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- 1989 Taylor, G.T. and S.K. Sharma. Advanced laser spectroscopy in marine sciences. In: Hawaii Institute of Geophysics Bi-annual Report, D. Henderson (ed.), pp. 12-15.
- 1989 Taylor, G.T., S.K. Sharma, B.E. Liebert, and H.F. Mower. Progress in molecular and microbiological investigations of marine biofouling at the University of Hawaii. ONR's Marine Biosurfaces Program Contractor's Mtg., May 20-22, 1989

In Preparation:

Taylor, G. T., P. J. Troy, M. Nullet, S. K. Sharma, B. E. Liebert and H. F. Mower. Examination of protein adsorption from seawater using FT-Infrared reflection-absorption spectroscopy, ellipsometry, and electrochemical impedance spectroscopy. Mar. Chem.

Troy, P., G. T. Taylor, M. Nullet, S. K. Sharma and B. E. Liebert. Adsorption kinetics and conformational changes of soluble protein on solid surfaces in seawater.

Taylor, G. T., G. Gyananath and D. Zheng. Adsorption of marine bacteria as a function of substratum surface properties.

Taylor, G. T., G. Gyananath and D. Zheng. Degradation of sorbed protein in seawater. Appl. Environ. Microbiol.

TRAINING ACTIVITIES:

This project has supported one Ph.D. candidate, Mr. Paul Troy, for three years and another Ph.D. candidate, Mr. Dongqiang Zheng, for 7 months. Both students are pursuing advanced degrees in Oceanography. The project also provided partial support for two M.S. candidates, Mr. Michael Nullet from the Department of Mechanical Engineering and Mr. John Wright (deceased) from the Department of Microbiology. Partial support was also provided for a postdoctoral associate, Dr. Garimella Gyananath visiting from the Biotechnology Centre of Marathwada University in Nanded, India.

Women or Minorities - 0

Non-Citizens - 2

AWARDS/FELLOWSHIPS:

None