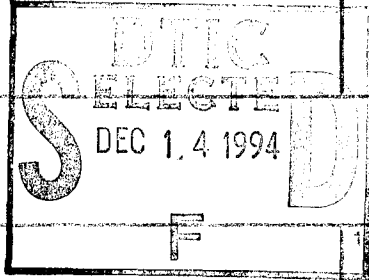


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Final Technical Report

ONR Grant Number N00014-93-1-1039

Title: Completion of Biofouling Research on the effects of
Marine Bacteria on the Attachment of Larval Barnacles

Introduction

The biofouling research performed as part of the present project, ONR grant N00014-93-1-1039 awarded to the South Carolina Wildlife and Marine Resources Department (SCWMRD), was both a continuation and extension of prior ONR-supported research (grant N00014-90-J-4048 awarded to South Carolina State University). In the prior project, laboratory studies of the effects of biofilms of three gram-negative rod bacteria, Deleya marina, Alteromonas macleodii, and Pseudomonas fluorescens, on attachment of barnacle cyprids were initiated. The attachment of cyprids of two barnacle species, Balanus amphitrite and B. improvisus, in response to bacterial films was studied, to compare the responses of the two species to biofilms and to assess the usefulness of each as a test organism in biofouling research. Research completed by the end of that project indicated that the responses of barnacle cyprids to bacterial cells and extracellular materials were variable and dependent upon the type of substratum to which the biofilms were attached. Because the scope and complexity of the initial ONR project

increased greatly from that originally proposed, additional funds were sought to bring the work to a logical conclusion.

An additional component of the original ONR-supported project was to recruit and train minority undergraduate students in marine science research through hands-on participation in all aspects of the project. Students participated in biofouling research and enrolled in marine science courses on the campus of South Carolina State University (SCSU) during the academic year. Summer training for the students included participation in research on the SCSU campus and at the Marine Resources Research Institute of SCWMRD. At least three of the fourteen students participating in the project have matriculated to graduate or professional schools in the life sciences. More information about past, present, and future activities on the part of SCWMRD to increase the number of minorities in marine science careers can be found in Appendix A.

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Objectives

The objectives of ONR contract N00014-93-1-1039 were:

- 1) To complete laboratory bioassays examining the effects of the bacteria Pseudomonas fluorescens, Deleya marina, and Alteromonas macleodii on attachment of larvae of the barnacles Balanus amphitrite and B. improvisus to polystyrene and glass surfaces.
- 2) To perform a study of the distribution and abundance of bacteria and their extracellular materials (ECM) on polystyrene and glass substrata.
- 3) To train an additional minority student in all aspects of the proposed research.
- 4) To complete statistical analyses and manuscript preparation summarizing findings from the research described above.

Technical Approach and Results

Objective #1: Complete laboratory bioassays

The methodology for laboratory bioassays of cyprid attachment in response to bacteria was similar in all assays. Bacteria grown in culture to mid-exponential (log) or stationary growth phase, or bacterial extracellular materials (ECM) produced during culturing, were exposed to sterile polystyrene petri dishes or baked (500°C for 4 h) borosilicate glass vials. Five milliliters of washed

bacterial cells, ECM, or sterile-filtered seawater (FSW) were added to the dishes and materials are allowed to attach for two or four hours. Substrata were rinsed free of unattached material, and the dishes or vials were refilled with 4.5 ml of filtered seawater. Approximately 25 barnacle larvae that recently molted to the cyprid stage were introduced to each dish or vial, and attachment of the cyprids was monitored at 24 h ($\pm$ 2 h) intervals for at least four days, to monitor changes in attachment behavior of cyprids over time. Assays were terminated by rinsing unattached cyprids from the substrata with distilled water and calculating the proportion that attached during each day of the assay.

Cells and extracellular material in culture supernatant from all three bacterial species had variable results on attachment of Balanus amphitrite cyprids: both enhancement and reduction of attachment was observed. In addition, the effects of the bacteria differed with substratum type employed in the assay. However, bacterial effects on larval attachment generally were consistent for the duration of an assay. These results were described in detail in a manuscript submitted to the journal Biological Bulletin (see Appendix B).

Larvae of Balanus improvisus responded in a more consistent manner to biofilms than did B. amphitrite larvae. All three species of bacteria generally decreased attachment of cyprids to polystyrene surfaces and increased attachment of cyprids to glass. The effect of Deleya marina on larval

attachment was also examined in field studies. Bryozoan larvae, but not barnacle cyprids, settled in greater numbers on polystyrene petri dishes to which *D. marina* cells were attached. However, dishes with bacterial ECM stimulated attachment of *B. improvisus* cyprids in the field, unlike results of laboratory assays. These results have also been summarized in manuscript format (Appendix B).

Objective #2: Examine the distribution and abundance of bacterial cells and ECM on polystyrene and glass substrata

It is critical to the interpretation of our experimental results, and the inconsistent results reported in the literature, to investigate causes of variability in attachment bioassays. We proposed to investigate variability in distribution patterns of bacterial cells and ECM on polystyrene and glass surfaces, as a possible cause of the variability of results of the larval attachment assays. We hypothesized that: 1) bacterial attachment and ECM adsorption may be nonuniform on a surface, causing patchy areas of attachment of cells or ECM, which vary in number within and among experiments; and 2) the total number of cells or concentration of ECM components attached to a surface may vary from one experiment to the next, even if the cells/ECM are relatively evenly distributed. We tested these hypotheses by staining bacterial cells and ECM to determine

whether they homogeneously coat the experimental substrata, and to estimate the concentration of adsorbed materials.

Bacterial cells from log and stationary phase cultures were allowed to attach to polystyrene petri dishes or glass microscope slides for four hours (Deleya marina and Pseudomonas fluorescens) or two hours (Alteromonas macleodii). Unattached materials were gently rinsed from the surfaces with sterile FSW and cells were fixed with Bouin's fixative. Cells were stained with ammonium oxalate-crystal violet, rinsed with distilled water, and dishes or slides were allowed to air dry overnight. Stained cells on the substrata were examined visually, and the intensity of the stain and its degree of uniformity on the surface were assessed qualitatively. Differences in cell staining patterns existed both among species and between substrata, and those differences were consistent in all assays performed. However, it was difficult to relate variability in assay outcome to patterns of biofilm thickness or evenness on polystyrene and glass substrata. Results of this work are described in a manuscript submitted to Biological Bulletin (see Appendix B).

Our first approach to staining ECM on polystyrene substrata was to use alcian blue, which stains acidic mucopolysaccharides. After bacterial ECM was allowed to adsorb to the dishes for two or four hours, the ECM was fixed with neutral buffered formalin. Dishes were rinsed with tap water then a 1% solution of alcian blue was added for 5-30

minutes. Dishes were then rinsed in running tap water and allowed to air dry. Dishes with ECM of all three bacterial species produced only a faint aquamarine color, although dishes with cells of Alteromonas macleodii and Deleya marina stained noticeably. Positive controls (nasal mucus smears) stained distinctively.

Our second approach to staining ECM was to employ the periodic acid-Schiff (PAS) staining method (Sigma test kit) for polysaccharides and mucoproteins. Bacterial cells or ECM were allowed to attach to petri dishes, then dishes were fixed with a formalin-ethanol solution and rinsed in tap water. Periodic acid was added to the dishes for 5 min, then dishes were rinsed in distilled water. Schiff's reagent was added to each dish for 15 min, then the dishes were rinsed with tap water and allowed to air dry. ECM of Alteromonas macleodii and Deleya marina did not produce a stain. However, cells of these species attached to petri dishes and stained in the same way produced a pale magenta color.

Our third approach in attempting to stain bacterial ECM for visualization of attachment patterns was to test the ability of bacterial cells and ECM to bind lectins. Two lectins were chosen for study, concanavalin A from jack bean (Canavalia ensiformis) and a lectin from wheat germ (Triticum vulgaris), based on their ability to bind to sugar groups commonly associated with bacteria. Both lectins, labelled with peroxidase, were purchased from Sigma Chemical Company.

Stationary phase bacterial cells and ECM were allowed to attach to polystyrene petri dishes as before. After rinsing, the dishes were refilled with sterile FSW and incubated overnight. The following morning, the FSW was decanted and then a small circle was marked on the bottom of each dish. To each circled area was added 100 μ l of 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for one hour, to decrease nonspecific binding by the lectin. After rinsing the circled area with PBS, 100 μ l of the lectin solution (0.5 mg ml⁻¹) or 100 μ l of the lectin in a 0.5 M solution of an inhibition sugar, was added to the circled area and incubated for 1 hour. After incubation, the circled area was rinsed three times with PBS then 100 μ l of a filtered solution of diaminobenzidine and hydrogen peroxide in Trizma base was added. After 20 min, the solution was rinsed off and the dishes were allowed to air dry.

ECM of Deleya marina did not bind either the con A or wheatgerm lectins to a noticeable degree at the concentrations used. Cells of this bacterial species produced a pale brown stain, indicating some binding of the lectins to the bacterial cells. Cells and ECM of Pseudomonas fluorescens produced a pale brown stain when the wheatgerm lectin was used. However, the wheatgerm lectin bound much more strongly to cells and ECM of Alteromonas macleodii, because a dark brown stain was produced.

None of the staining procedures tested produced results sufficient in power or consistency to test the hypothesis of

spatially variable adsorption of ECM to surfaces. Therefore, a different approach was used to document that bacterial ECM did in fact adsorb to the polystyrene and glass surfaces used in the larval barnacle attachment assays. We used a method measuring the contact angles water droplets make on surfaces, to provide an indication of surface wettability of control surfaces compared to those with bacterial cells or ECM adsorbed. Details of this method and the results obtained are described in a manuscript submitted to Biological Bulletin (Appendix B). Bacterial cells and ECM on polystyrene caused water droplets to have smaller contact angles than droplets on control surfaces indicating an increase in wettability, whereas cells and ECM on glass caused the contact angles to increase over control values, indicating a decrease in wettability of the surface.

Objective #3: Training of an additional minority student

Unfortunately, ONR funding was not available in time for a summer intern to become trained in all aspects of the proposed work. However, two African American students working as volunteers participated in several aspects of the project. Iris Nelson, a biology major at the University of South Carolina, volunteered in the laboratory for 1 day a week during the summer of 1993. She helped in analysis of the larval attachment assays, cell staining, and with obtaining the contact angle measurements. Tiffany Mowatt, a

freshman at James Island High School, assisted with the cell staining work during the fall of 1993.

Objective #4: Completion of statistical analyses and manuscript preparation

All statistical analyses were completed. Three manuscripts based on ONR-supported research were completed (see Appendix B). One manuscript is in press in Journal of Experimental Marine Biology and Ecology. Another manuscript was submitted to Biological Bulletin. The third has been reviewed by scientists at the South Carolina Marine Resources Research Institute and will be submitted to Journal of Experimental Marine Biology and Ecology. In addition, an article describing the training program in marine science for minority students was published in "The Resource", the newspaper of SCWMRD.

Summary of Research Results

Our present and prior ONR-funded research has demonstrated that:

- marine bacteria affect attachment of larval barnacles
- the nature of the surface to which bacteria are attached influences the type of effect (negative or positive) that bacteria have on larval attachment
- the variability of results is not readily explained by variability in patterns of bacterial cell attachment
- attachment assays employing Balanus improvisus cyprid larvae are more predictable and consistent in outcome than are those using B. amphitrite as the test organism

Appendix A: ONR-supported minority training in marine science

During the three years (1990-1993) of the cooperative training program for minorities in marine science at South Carolina State University (SCSU) and the South Carolina Wildlife and Marine Resources Department (SCWMRD), staff and students at both institutions learned valuable lessons regarding strategies to facilitate success in minority training programs. SCWMRD staff learned the value of hands-on, mentor-based student learning experiences as a training approach. The level of student involvement in conducting research and developing research products must be high in order to stimulate and reinforce student interest in research. Students learned the importance of applying themselves to a rigorous academic science curriculum to successfully compete for future careers in science.

The ONR program was very successful in introducing many undergraduate students to marine and environmental science as an alternative career opportunity, and in interesting several students in continuing their education in marine and environmental science. The program also was successful in establishing strong ties between faculty, research, and administrative staff at both SCWMRD and SCSU. The demonstration of the feasibility of a partnership between a historically black state university and a state resource management agency has created interest in many other agencies

at the state and federal levels in becoming part of this minority training partnership.

Direct results of the success of the ONR minority training program include the increase in marine and environmental science activities undertaken by SCWMRD and directed toward minorities, initiation of a mentor-based summer intern project, and the establishment of a minority affairs committee within MRD. Recruitment efforts have been greatly increased, whereby several Historically Black Colleges and Universities in South Carolina and Georgia are targeted for regular visitation by SCWMRD staff to 1) meet with department heads and administrators, 2) present classroom seminars to introduce the program to faculty and students, and 3) identify interested students. Experience gained from the ONR project will aid SCWMRD in defining approaches to expand future training projects. A combination of multi-institutional resources on a regional level with multiple funding sources should increase the probability of success in meeting the long-term goal of encouraging and training minorities in careers in the marine and environmental sciences. Future projects at MRD will attempt to establish strong cooperative interactions among many South Carolina agencies to create a comprehensive educational experience for minorities interested in marine or environmental science. The knowledge students gain will assist them in making career decisions, and provide them with skills necessary for successful performance in graduate and professional schools,

in scientific fields relevant to the U.S. Navy and the Office of Naval Research.

Students of the Office of Naval Research Program

Name of Student	Time in ONR Program	Current Status
Melissa Sargent	June 90-May 91 (12 mo)	B.S., Biology, SCSU Employed at Roche Pharmaceuticals - NC
Arve Hammock	June 90-Dec 90 (7 mo)	B.S. Chemistry, SCSU Employed at Vintage Pharmaceuticals - NC
Brenda Frazier	June 90-May 91 (12 mo)	B.S., Biology, Employed by Dr. John Williams, Ecologist, SCSU, (14 mo); Currently working in Texas
Alexis Ware	June 91-July 92 (14 mo)	B.S., Biology, SCSU; Completed 93 course at Duke Univ. Mar. Lab; Currently enrolled in the M.S. program in Wildlife and Fisheries at Tenn. Tech
Shauna Patterson	June 91-July 92 (14 mo)	Biology major - SCSU
Dionne Sumpter	June 91-Nov 91 (6 mo)	Biology major - SCSU
Tammie Terrell	June 91-Oct 91 (5 mo)	B.S., Biology, SCSU
Chenise Lambert	June 91-July 91 (2 mo)	Biology major - SCSU
Dawn Evans	July 91-Oct 91 (4 mo)	B.S., Biology, SCSU; Employed with Ethyl Chemical Plant, SC
Natombi Smith	June 91-May 92	B. S., Biology, SCSU; Enrolled in M.S. program at Xavier of Ohio in Marine Science
Leyana Lloyd	June 91-Jan 93 (20)	Chemistry major - SCSU
George McCowan	June 92-Nov 92 (8 mo)	Biology major - SCSU Spring 93 internship at MRD
Michelle Haynes	June 92-May 93 (11 mo)	Biology major - SCSU

Dhayalini Chandrasegaram	June 92-May 93 (11 mo)	B.S. Biology/Chemistry Claflin College
Valinda Olds	April 93-May 93	Biology Ed. major Claflin College
Nalani Kalawe	April 93-May 93	Psychology major - SCSU
Scottie McDuffie	April 93-May 93	Biology major - SCSU
Aliyah Spruill	April 93-May 93	Biology major - SCSU

Appendix B: Publications resulting from ONR-supported
biofouling research and minority training*

O'Connor, N.J. and D.L. Richardson. In press. Comparative attachment of barnacle cyprids (Balanus amphitrite Darwin, 1854; B. improvisus Darwin, 1854; & B. eburneus Gould, 1841) to polystyrene and glass substrata. J. Exp. Mar. Biol. Ecol.

O'Connor, N.J. and D.L. Richardson. Submitted to Biol. Bull. The influence of surface-associated bacteria on attachment of barnacle (Balanus amphitrite) cyprids.

O'Connor, N.J. and D.L. Richardson. In prep. Effects of bacteria on attachment of barnacle cyprids (Balanus improvisus): laboratory and field studies.

O'Connor, N.J. 1993. Minorities encouraged to enter marine science. "Wildlife and Marine Resources", the newspaper of the South Carolina Wildlife and Marine Resources Department, Spring edition.

*reprints of all publications can be obtained by contacting:

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