REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.
PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
25/05/2020	Final	15/09/2017-14/03/2020

4. TITLE AND SUBTITLE
Multiple Approaches for Evaluating Novel Marine Coatings in the Pearl Harbor Naval Base and in the Laboratory

5a. CONTRACT NUMBER

5b. GRANT NUMBER
N00014-17-1-2979

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Hadfield, Michael, G
Nedved, Brian, T

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Hawaii
Office of Research Services
2425 Campus Rd Sinclair Rm 1
Honolulu, HI 96822-2247

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research
875 N. Randolph St., Suite 1425
Arlington VA 22203

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for Public Release; Distribution is Unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
We have continued to test emergent hull coatings at Pearl Harbor, Hawaii. Our test site at Ford Island is at full capacity with over 300 experimental coatings from 5 ONR-funded researchers. In an attempt to further understand the mechanisms that cause recruitment of biofouling invertebrates onto submerged surfaces, we have isolated additional strains of bacteria that induce metamorphosis of the problematic biofouler Hydroides elegans and determined that surface associated lipids act as inductive molecules. Finally we have used metagenomic analysis to determine the relative abundance and diversity of bacteria in biofilms from Pearl Harbor. Having an estimation of these data will aid in the development of novel coatings.

15. SUBJECT TERMS
Field Testing of Experiemental Coatings
Larval Inductive Cues
Metagenomics

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Michael G. Hadfield
			UU	9	19b. TELEPHONE NUMBER *(Include area code)* 808-539-7319

Final Technical Report
GRANT # : N00014-17-1-2979
PRINCIPAL INVESTIGATOR : Michael G. Hadfield and Brian T. Nedved
INSTITUTION: University of Hawaii at Manoa
GRANT TITLE : Multiple Approaches for Testing Novel Marine Coatings in the Laboratory and in Pearl Harbor
AWARD PERIOD: 09/15/17-3/14/20

OBJECTIVE : To provide rapid testing of emergent foul-release and anti-fouling hull coatings on panels deployed in the tropical harbor at Pearl Harbor, Hawaii; to provide rapid and precise field and laboratory testing of experimental anti-fouling coatings as acceptable substrates for recruitment of bacteria and larvae of the fouling species *Hydroides elegans*, a calcareous-tube worm; to provide rapid and precision testing of experimental foul-release coatings in the turbulent flow cell to determine shear forces necessary to remove fouling organisms; and to use molecular tools to determine identities of bacterial species in biofilms that induce the settlement of the tubeworm *Hydroides elegans* and other fouling invertebrates.

APPROACH: (1) We provided tests at our Ford Island test site of new coatings following existing protocols for ASTM-based visual inspections, removal of slimes and soft foulers via water jet and force gauge measurements. These methods allow for comparisons of the effectiveness of coatings as well as the effectiveness of these coatings over time. **(2)** We provided rapid laboratory testing of experimental antifouling and foul-release coatings. To accomplish this, we first suspend the coated slides in a sea table at the marine laboratory that receives a continuous flow of natural, unfiltered seawater for 10 – 14 days during which time the slides become coated with a natural bacterial film. This film is necessary to induce the settlement of larvae of the biofouling tubeworm *H. elegans* onto the coatings. Larvae are reared in the laboratory according to procedures we have developed; the larvae are competent to settle five days after fertilization of eggs collected form adults brought from the field. When we have prepared the competent larvae, we expose the coated and biofilmed slides to the larvae overnight and determine how many settled on the slides by direct count. Once the biofilmed slides are colonized by the tubeworms, the slides are placed into the turbulent flow cell in our lab and subjected to increasing shear forces from flowing seawater, and the force required to remove them is recorded. **(3)** Previous results showed that the common marine biofilm bacterium *Pseudoalteromonas luteoviolacea* is a major inducer of recruitment of the calcifying tubeworm *Hydroides elegans*. Using the tools of molecular genetics, we have determined a genetic basis for generation of the inducing factor by this bacterium. we expanded on this research by determining the identities of other inductive bacterial species in Hawaiian harbors and learning if they induce recruitment of biofouling organisms by the same mechanism or a different one.

ACCOMPLISHMENTS:

1. Testing of emergent foul-release and anti-fouling hull coatings in the field

We have continued to test emergent foul-release and anti-fouling hull coatings at Pearl Harbor, Hawaii. Our test site was at full capacity for much of the grant period with over 336 experimental panels with 86 different coating from six ONR-funded researchers (Fig.1A). For the coatings in our testing array, coating formulation X7 from Hempel paints (Holm's intersite calibration set) showed excellent anti-fouling properties and continues to remain free of macrofoulers throughout the test period (Fig. 1B). Further, we have established a new protocol for estimating percent cover using the program PhotoQuad.

2. Laboratory testing of experimental anti-fouling and foul-release coatings

During the 2017 – 2020 testing period, coated slides were not submitted to our testing facility by any ONR contractor although numerous email solicitations were sent. In the event that our testing facility receives coated slides from any ONR contractor, we

measure their resistance of the coatings to fouling by the tubeworm *Hydroides elegans* through the use of a laboratory settlement assay and then measuring the forces necessary to remove the settled worms in our turbulent flow cell. Additionally, we have developed a protocol to use the solitary tunicate *Phallusia philippinensis* as a laboratory model for testing the forces required for the removal of soft foulers from experimental foul-release coatings. The larvae of *P. philippinensis* settle and metamorphose rapidly, and, because they will metamorphose on clean surfaces, can be forced to settle on a wide variety of experimental surfaces.

3. Using molecular tools to determine bacterial products that induce metamorphosis of marine invertebrate larvae

We have previously isolated several strains of bacteria that produce compounds that induce larvae of the serpulid polychaete *Hydroides elegans* to rapidly undergo metamorphosis. One of these strains of bacteria, *Pseudoalteromonas luteoviolacea*, produces a phage-tail bacteriocins when it is in a biofilm and these phage-tail bacteriocins are potent metamorphic inducers for larvae of *H. elegans*. We have isolated three additional strains of inductive bacteria from biofilms from our Ford Island test site. These strains have been identified as the gram-negative bacterium *Cellulophaga lytica* and two gram-positive bacteria, *Staphylococcus warneri* and *Bacillus aquimaris*. Single-species biofilms from all three of these strains induce metamorphosis, and we made cell-free extracts from overnight cultures of all three of these bacteria. These extracts also proved to have inductive properties for larvae of *H. elegans* (Fig. 1C). In an attempt to determine if phage-tail bacteriocins are produced by these inductive strains, we used both molecular techniques and bioinformatic approaches to search for homologues of phage-tail bacteriocins. We have examined these extracts using transmission electron microscopy (TEM) and could not find any phage-tail bacteriocins within them. We also used ultracentrifugation to concentrate the inducer within the cell-free extracts from these three bacterial strains, and examination of the resulting pellet using TEM showed that they contained numerous extracellular vesicles (Fig. 1D). Further, we searched the genomes of all three of these inductive strains for homologues of the genes that encode for phage-tail bacteriocins and the genomes of *C. lytica, S. warneri,* and *B. aquimaris* do not contain these genes.

Outer membrane vesicles isolated from *C. lytica* induce larvae of H. elegans to metamorphose. HPLC and mass spectrometry was used to to gain an understanding of the biochemical nature of the OMV-associated compounds that induce metamorphosis. Additionally, we began subjecting OMV fractions to an array of enzymatic tests (DNase, RNase, proteinase K and heat). To date, none of these tests has reduced the metamorphic activity of the OMV fraction.

We subsequently employed lipases and lysozyme to determine if either the membrane layer or the peptidoglycan portion of the OMV was acting as an inducive cue for larvae of *H. elegans*. Treatment of the OMVS with lysozyme did not alter their inductive capabilities. These results suggested that peptidoglycan from *C. lytica* was not acting as an inductive cue. Treatment of OMVS with lipases did significantly reduced the inductive capabilities of OMV. A general lipase enzyme, as well as the enzymes phospholipase A1, phospholipase A2, and phospholipase C all significantly degraded OMVs as a metamorphic cue.

From these new experiments we deduced that OMVs from *C. lytica* are active only because they include pieces of the cell envelope. i.e., a 'cargo' is not implicated. Finally, we determined through isolation and purification of the components of the cell envelope that LPS is most likely the molecule of interest for *C. lytica*-induced metamorphosis of *H. elegans*.

Additionally, we have performed Illumina sequencing of bacterial 16S ribosomal DNA to analyze the composition of bacterial communities within microbial biofilms isolated from our Ford Island site and to compare these communities to other marine bacterial communities around Oahu. Our results suggest that well over fifty different dominant strains of bacteria comprise the community within biofilms from Pearl Harbor. The community composition of these biofilms is also quite different than the community of bacteria present in the surrounding seawater. This biofilm community from Pearl Harbor is also different than bacterial biofilms from other habitats around Oahu. We have also searched these metagenomes for the molecular fingerprint of our four inductive strains of bacteria and they are found in very low abundance in the biofilms from Pearl Harbor. These results may suggest that numerous different bacterial strains are producing similar types of inductive cues (i.e. LPS) and biofouling organisms are induced to metamorphose by these cues regardless of the strain of bacteria that produced them. However, a more interesting mechanism may be that even though there are diverse bacterial communities within microbial biofilms, it is rarely occurring strains of bacteria that produce a highly specific cue that induces metamorphosis of larvae of biofouling organisms. It will be of great interest to isolate bacterial strains that are common members of the biofilm community at Pearl Harbor but are non-inductive, and then analyze the structure of the LPS that decorate the outsides of the bacterium. Comparing structural differences between the LPS of inductive and non-inductive strains may help to clarify if larvae of biofouling organisms respond to sets of ubiquitously expressed cues or very specific ones from rarely occurring bacteria within a biofilm. These conclusions can be of great use in the development of novel anti-fouling coatings because knowledge of the nature of the metamorphic cues from bacteria may allow chemists within the ONR group to directly target and disrupt these cues.

CONCLUSIONS *:* We have continued to test emergent foul-release and anti-fouling hull coatings at Pearl Harbor, Hawaii. Our test site at Ford Island is at full capacity with over 300 experimental coatings from 5 ONR-funded researchers.

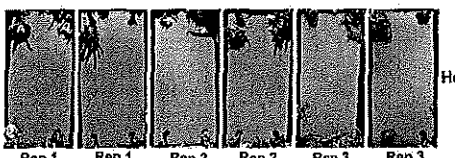
Further, in an attempt to further understand the mechanisms that cause recruitment of biofouling invertebrates onto submerged surfaces, we have isolated additional strains of bacteria that induce metamorphosis of the problematic biofouler *Hydroides elegans* and have begun to investigate the composition of these bacterial cues. Single-species biofilms composed of the previously isolated gram-negative bacterium *Cellulophaga lytica* and the gram-positive bacteria *Bacillus aquimaris* and *Staphylococcus warneri*, induce metamorphosis of *H. elegans*. Both *in silico* and *in situ* analyses demonstrate that the inductive products produced by these bacteria are not tailocins but may be surface associate lipids (either LPS for Gram-negative bacteria or lipoteichoic acid for Gram-positive bacteria). Tailocins are the inducers produced by the bacterium *Pseudoalteromonas luteoviolacea*. These results suggest that there may be multiple mechanisms by which biofilm bacteria produce inductive cues. Finally, we are using

metagenomic analysis to determine the relative abundance and diversity of bacteria in biofilms from Pearl Harbor. Having an estimation of the diversity of both bacterial species and the inductive cues they produce will aid in development of novel coatings.

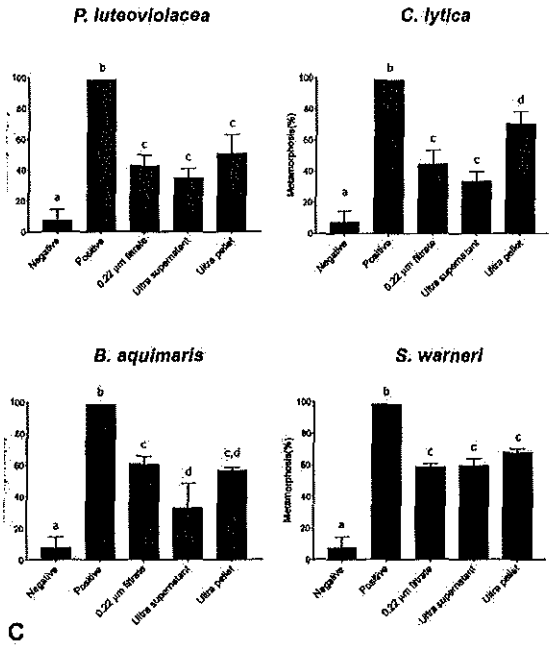
SIGNIFICANCE : The accumulation of marine microorganisms and attached animals and plants on the hulls of ships, the surfaces of pilings and floating docks and the interiors of pipes that provide cooling water to electrical plants and other industries costs the U.S. Navy many millions of dollars per year for added fuel costs and cleaning. While it has long been known that the first step in marine biofouling is the accumulation of microbial films on newly submerged surfaces, it has only been in the last 20 – 30 years that we have learned that cues from biofilms are the predominant stimulus for settlement of the marine invertebrate animals –barnacles, sponges, tube worms, oysters, etc.– that are the major problem in biofouling. Our most recent research on a single bacterial species that induces recruitment of the major problem fouler *Hydroides elegans*, as well as other marine species, used genomic and biochemical approaches to discover that LPS produced by the bacterium is the inductive agent. While now poised to understand at much greater depth the bacterial basis of biofouling and with the methods available to probe these questions, the proposed research has the potential to finally broadly explain the stimulus-and-response relationship between biofilm bacteria and the massive, problematic biofouling that they cause. With this understanding will come novel insights into approaches to prevent biofouling at its most incipient level and thus save the U.S. Navy many millions of dollars and thousands of hours of lost time while large ships are dry-docked for hull scraping and recoating.

INSTITUTION	Contact Scientist	# Surfaces	COATING TYPE
Harvard	Alzenberg	110	FOUL/RELEASE ANTIFOULING
NSWC	Holm	96	FOUL/RELEASE ANTIFOULING
NDSU	Stafslien	40	FOUL/RELEASE ANTIFOULING
NDSU	Webster	64	FOUL/RELEASE ANTIFOULING
SUNY Buffalo	Detty	8	FOUL/RELEASE ANTIFOULING
Nature Coat	Xu	18	FOUL/RELEASE ANTIFOULING
		336 TOTAL SURFACES	

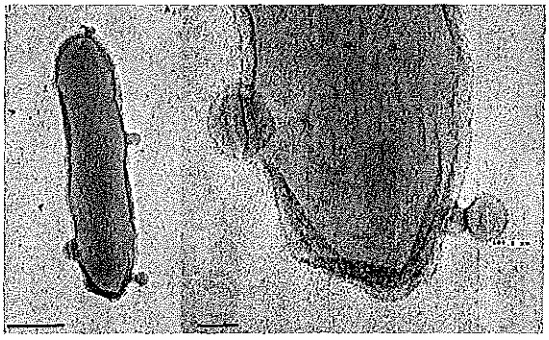
A



B



C



D

Figure 1. Summary of testing 2017-2020. A. Coatings supplied by ONR-affiliated groups B. Coating X7 from Hempel performs well as an antifouling coating C. Isolated bacterial strains that induce metamorphosis of *H. elegans* D. OMVs from *C. lytica*

PATENT INFORMATION : None

AWARD INFORMATION :

PUBLICATIONS and ABSTRACTS (for total period of grant) :

Publications

Freckelton, M., B. T. Nedved and M. G. Hadfield. 2017. Induction of Invertebrate Larval Settlement; Different Bacteria, Different Mechanisms? Scientific Reports, DOI: 10.1038/srep42557.

Summers, S., M. Freckelton, B. Nedved, S. Rice, and M.G. Hadfield. 2019. The full Genome sequence of Thalassotalea euphylliae H2 strain. Microbiology Resource Announcements, DOI: 10.1128/MRA.01608-18.

Vijayan, N., K. A. Lema, B. T. Nedved, and M. G. Hadfield. 2019. Microbiomes of the polychaete Hydroides elegans (Polychaeta: Serpulidae) across its life-history stages. Marine Biology, 166:19. DOI: 10.1007/s00227-019-3465-9.

Vijayan, N. and **M. G. Hadfield**. 2020. Characterizing the microbial diversity of a natural biofilm that induces larval settlement of *Hydroides elegans*. Aquatic Microbial Ecology, 84:31-42. doi.org/10.3354/ame01925.

Freckelton, M., and B. T. Nedved. 2020. When does symbiosis begin? Bacterial cues necessary for metamophosis in the marine polychaete *Hydroides elegans*. in *Cellular Dialogues in the Holobiont*, T. C. G. Bosch and M. G. Hadfield, eds. CRC Press, Boca Raton, FL.

Freckelton, M. L., Nedved, B. T., Cai, Y.-S., Cao, S., Turano, H., Alegado, R. A. and Hadfield, M. G. 2020. Bacterial lipopolysaccharide induces settlement and metamorphosis in a marine larva. BioRXiv. doi.org/10.1101/851519 (submitted to PNAS 12/03/2019; returned 1/21/2020 for revision).

Invited Symposia or Seminars:

Invited

2017. M. G. Hadfield, invited participant: "Symbiotic Interactions in the Oceans," January 23 – 27, 2017, Maui, HI. A workshop sponsored by the Canadian Institute for Advanced Research and the Gordon and Betty Moore Foundation; Maui, Hawaii.

2018. M. G. Hadfield, invited seminar: "Metamorphosis of Marine Invertebrates: a primer." Tropical Marine Science Institute, Singapore National University, Singapore. April 5, 2018.

2018. M. G. Hadfield, invited seminar: "The bacterial basis of larval settlement: a case study, *Hydroides elegans.*" Smithsonian Marine Station, Ft. Pierce, FL. June 2018.

2019. M. G. Hadfield. What have we learned from the primary biofoulers, the bacteria? Office of Naval Research, Annual Review, Special Session. June 2019.

Contributed papers/talks:

Society for Integrative and Comparative Biology, January 2016, Portland, OR. M.G. Hadfield and B. T. Nedved "The bacterial basis of larval settlement: a case study, *Hydroides elegans.*"

Society for Integrative and Comparative Biology, January 2016, Portland, OR. G. Batzel, B.T. Nedved, and M.G. Hadfield, "Presence and localization of carbonic anhydrase genes in *Hydroides elegans.*"

Society for Integrative and Comparative Biology, January 2016, Portland, OR. B.T. Nedved, G. Batzel, and M.G. Hadfield "Molecular analysis of tube cement of the biofouling tubeworm *Hydroides elegans.*"

Western Society of Naturalists, November 2016, Monterey, CA, M.G. Hadfield "The bacterial basis of larval settlement."

Society for Integrative and Comparative Biology, January 2017, New Orleans, LA, "Bacterial genomes and larval settlement: are predictions possible?"

Society for Integrative and Comparative Biology, January 2017, New Orleans, LA, "Multiple bacterial cues induce larval invertebrate settlement."

11th International Larval Biology Symposium, August 2017, Honolulu, HI, M.L. Freckelton, K. Lema, B.T. Nedved, and M.G. Hadfield. "Multiple microbes, multiple mechanisms: bacterial induction of *Hydroides* settlement."

11th International Larval Biology Symposium, August 2017, Honolulu, HI, M.A.R. Koehl and M.G. Hadfield. "How can larvae tumbling in turbulence enhance their settlement in different flow habitats?"

11th International Larval Biology Symposium, August 2017, B.T. Nedved, M.L Freckelton, and M.G. Hadfield, Honolulu, HI, "Bacterial Genomes and Larval Settlement: Are Predictions Possible?"

Society for Integrative and Comparative Biology, January 2018, San Francisco, CA, B.T. Nedved. "Bacterial induction of metamorphosis of *Hydroides elegans* (Polychaeta): a new twist in the tailocin tail." January 3-7, 2018.

Society for Integrative and Comparative Biology, January 2018, San Francisco, CA,M.G. Hadfield. "Metamorphosing larvae of *Hydroides elegans* (Polychaeta): the first 30 minutes on the bottom."

Society for Integrative and Comparative Biology, January 2018, San Francisco, CA, 2018. M. L. Freckelton. "Searching for the mechanism: enzymatic interrogations of outer membrane vesicles involved in the metamorphosis of *Hydroides elegans.*"

Australian Microbial Ecology Conference, Perth, Western Australia, Australia, February 2019. M.L. Freckelton. "Understanding settlement: the role of common bacterial

biofilm elements in the induction and metamorphosis of the polychaete *Hydroides elegans."*