REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
25-09-2015	Final Report	01-05-2015 to 30-04-2015

4. TITLE AND SUBTITLE
Multiple Approaches for Testing Novel Coatings in the Laboratory and in Pearl Harbor, Hawaii with Emphasis on the Global, Problem-Fouling Invertebrates

5a. CONTRACT NUMBER

5b. GRANT NUMBER
N00014-11-1-0167

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

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Seattle, WA 98104

10. SPONSOR/MONITOR'S ACRONYM(S)
DOD/ONR

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

12. DISTRIBUTION/AVAILABILITY STATEMENT

13. SUPPLEMENTARY NOTES

14. ABSTRACT
We performed field tests on 111 panels with 33 different coatings from four ONR-affiliated laboratories. The resultant data have been reported back to the contractors, who are making additional changes to their compounds for further testing. Investigations of the bacterial basis of recruitment of larvae of biofouling animals to marine surfaces have found new bacterial mechanisms for three new species isolated from biofilms in Hawaii and identified with molecular genetics.

15. SUBJECT TERMS
Biofouling, Hydroides elegans, Settlement, Metamorphosis, Bacterial Biofilm.

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
					19b. TELEPHONE NUMBER *(Include area code)* 808-539-7319

Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std. Z39.18

FINAL TECHNICAL REPORT

Review Period: 1 May, 2014 – 30 April, 2015

TITLE: Multiple Approaches for Testing Novel Coatings in the Laboratory and in Pearl Harbor, Hawaii with Emphasis on the Global, Problem-Fouling Invertebrates

ONR AWARD NUMBER: N00014-11-1-0167

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a. Scientific and Technical Objectives:

To provide rapid testing of novel foul-release and anti-fouling hull coatings on panels deployed in the tropical harbor at Pearl Harbor, Hawaii; to provide rapid and precision laboratory testing of experimental anti-fouling coatings as acceptable substrates for recruitment of larvae of fouling species; 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 (*Hydroides elegans*); to develop novel methods for the propagation/storage of H. *elegans*.

b. Technical Approach:

(1) We field tested coatings following existing protocols for ASTM-based visual inspections, water jet determinations and force gauge measures. **(2)** We evaluate coatings for removal of foulers in the flow cell. **(3)** We examined removal forces for *Hydroides elegans* from coated slides in an apparatus that allows forces for removal of submerged animals to be monitored with a motorized, very evenly applied increasing force. **(4)** We continued to analyze marine biofilms to isolate bacterial species that induce recruitment of *H. elegans* to marine surfaces and used molecular approaches to determine full genomes of two inductive bacterial species, *Pseudoalteromonas luteoviolacea* and *Cellulophaga lytica* and analyzed the genomes to determine the nature of the products that are responsible.

c. Concise Accomplishments:

May 1, 2014 – April 30, 2015

Abstract: We performed field tests on 111 panels with 33 different coatings from four ONR-affiliated laboratories. The resultant data have been reported back to the contractors, who are making additional changes to their compounds for further testing. Investigations of the bacterial basis of recruitment of larvae of biofouling animals to marine surfaces have found new bacterial mechanisms for three new species isolated from biofilms in Hawaii and identified with molecular genetics.

d. Expanded accomplishments:

May 1, 2014 – April 30, 2015

(1) Field Testing

The following table summarizes the field testing of coated panels and laboratory testing of coated slides (calibrated flow cell) submitted to us by ONR contractors during the granting period (2014-2015). Some of these tests are on-going.

INSTITUTION	PRINCIPAL INVESTIGATOR	# PANELS	COATING TYPE
NSWC	HOLM	24	FOUL/RELEASE ANTIFOULING
NORTH DAKOTA STATE UNIVERSITY	WEBSTER	32	FOUL/RELEASE ANTIFOULING
UNIVERSITY OF WASHINGTON	JIANG	30	FOUL/RELEASE ANTIFOULING
TEXAS A&M UNIVERSITY	WOOLEY	16	FOUL/RELEASE ANTIFOULING
ZWITTER TECHNOLOGY, LLC	LI	9	FOUL/RELEASE ANTIFOULING
		111 TOTAL PANELS	

(2) Laboratory testing – Calibrated Flow Cell

No ONR contractors submitted slides/coupons with experimental coatings for evaluation in the turbulent flow cell during the calendar year of this grant.

(3) Investigating bacterial induction of larval settlement (2014-2015)

Our report in Science in 2014 (Shikuma *et al.* 2014) was the first to describe in the biofilm bacterium *Pseudoalteromonas luteoviolacea* a phage tail-like component that is capable of inducing the metamorphosis of a marine invertebrate. However, our continued studies in the current year have revealed that a role for bacteriocins is apparently not wide spread. We have recently gained evidence that another common and inductive biofilm bacterium, *Cellulophaga lytica* (Huang and Hadfield, 2003) does not produce bacteriocins. We have obtained the genome sequence of this inductive bacterium by 3rd Generation PacBio SMRT long-read sequencing methods (Asahina and Hadfield, 2014). Assembly and annotation of the coding regions with RAST (Rapid Annotation Subsystem Technology) webserver revealed that *C. lytica* does not contain genes encoding for essential proteins of bacteriocins found by Shikuma *et al.* (2014) to induce metamorphosis in *H. elegans*. Additionally, we have evidence from transmission electron microscopy (TEM) that *C. lytica* produces large quantities of outer membrane vesicles (OMV) (Fig 1). OMVs arise from outpocketings of the bacterial plasma membrane (Figs. 1A&B), and cell-free filtrates of *C. lytica* are devoid of bacterial cells yet still induce metamorphosis of *H. elegans*. OMVs are constitutively expressed by gram-negative bacteria but can be products of gram-positive bacteria as well (Dorward *et al.*, 1990; Lee *et al.*, 2009; Rivera *et al.*, 2010). OMVs have also been described for the marine bacteria *Shewanella spp.* (Gorby *et al.*, 2008) and *Pseudoalteromonas antarctica* (Nevot *et al.*, 2006). OMVs have gained particular interest recently due to their ability to contain a variety of compounds including bacterial lipids, outer membrane proteins, periplasmic content, and other insoluble components that are important in pathogenesis, interspecies communication, biofilm formation, nutrient acquisition, and DNA transfer (Beveridge *et al.*, 1996; Beveridge *et al.*, 1997; Davies *et al.*, 1998; Ciofu *et al.*, 2000;

Amano *et al.*, 2010; Berleman and Auer, 2013; Biller *et al.*, 2014: Bonnington and Kuehn, 2014). Finally, we have isolated two gram-positive biofilm bacterial species that induce settlement and metamorphosis of *Hydroides elegans* and, again, found their genomes to lack components of inductive phage-tail bacteriocins. Based on DNA sequences of the 16s ribosomal gene, these sepcies are *Bacillus aquimaris* and *Staphylococcus warneri.*

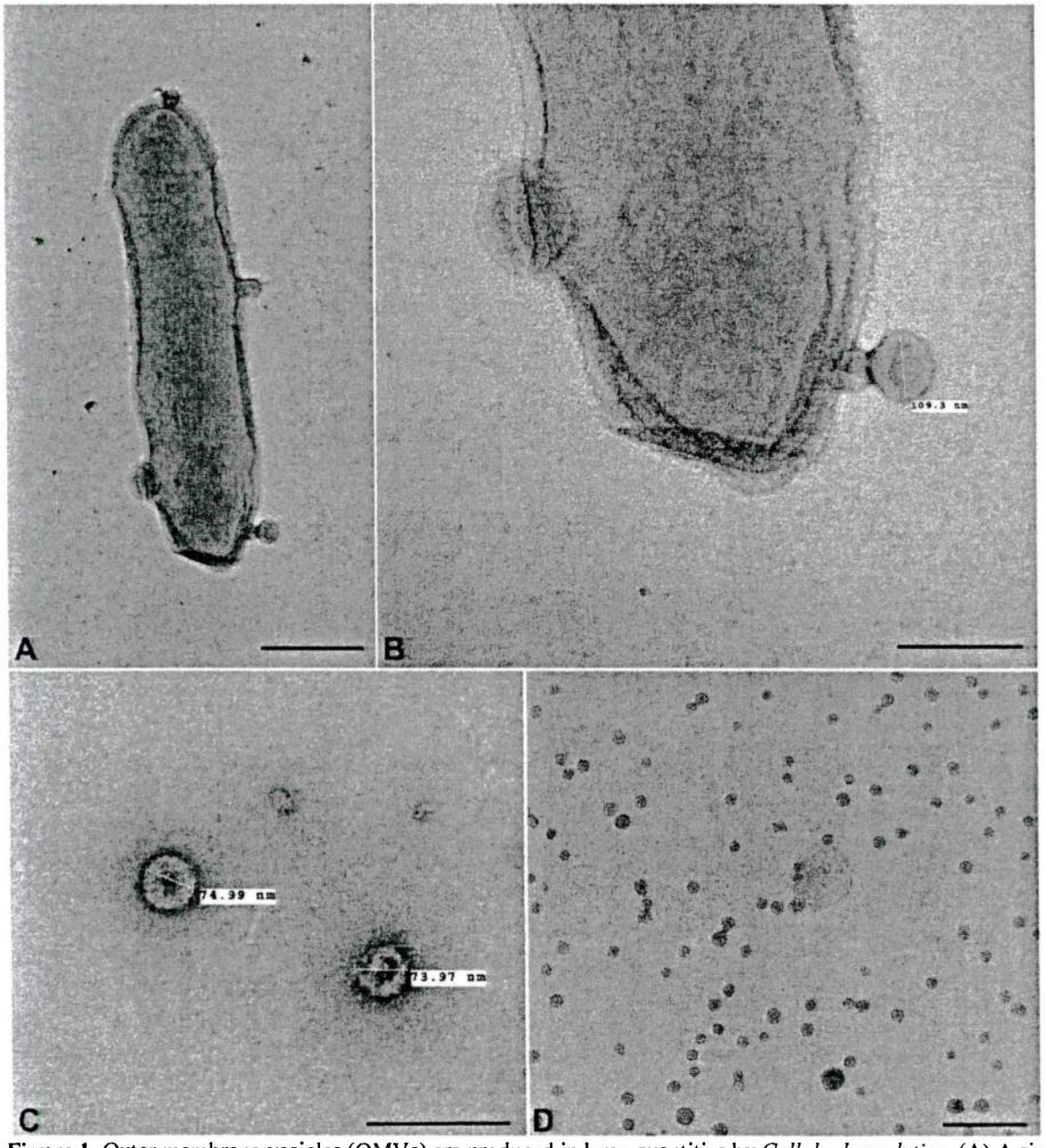

Figure 1. Outer membrane vesicles (OMVs) are produced in large quantities by *Cellulophaga lytica.* (A) A single bacterium produces several OMVs; scale bar = 500 nm. (B) OMVs are produced as out-pocketings of the bacterial membrane; scale bar = 200 nm. (C) Individual OMVs are about 75-100 nm in diameter; scale bar = 200 nm. (D) Numerous OMVs are present in cell-free filtrates from broth cultures of *C. lytica.*; scale bar =500 nm **Figure 3.** OMVs in cell free-filtrates from cultures of *Cellulophaga lytica* induce metamorphosis of *Hydroides elegans.*

e. Work Plan.

This is the Final Report

f. Major Problems.

No major problems

h. Foreign Collaborations.

None

Papers published:

Asahina, A. Y., and M. G. Hadfield. 2014. Complete genome sequence of *Cellulophaga lytica* HI1 using PacBio single-molecule real-time sequencing. genomeA, 2(6): 1-2 (e01148-14).

Hadfield, M. G., A. Asahina, S. Hennings and B. Nedved. 2015. The bacterial basis of biofouling: a case study. Indian Journal of Geomarine Science, 43 (11): 2075-2084.

Asahina, A. Y. and M. G. Hadfield. 2015. Draft Genome of *Pseudoalteromonas luteoviolacea* HI1 using Roche 454 and PacBio Single Molecule Real-Time Hybrid Sequencing. Genome Announcement 3(1): e01590-14. doi:10.1128/genomeA.01590-14.

Presentations:

'Symbiomics,' 10-day workshop by the Marine Microbiology Institute, Max Planck Inst., Bremen, Germany. Invited faculty participant: two lectures and project leader. Held at the Hydra Laboratory, Isle of Elba, Italy, May 27 – June 7, 2014.

Academia Sinica, Biodiversity Research Center, Taipei, Taiwan. Invited symposium speaker: "The bacterial basis of marine biofouling." April 9, 2015.

Canadian Institute for Advanced Research, Symposium on Integrated Microbial Diversity. Invited speaker: "The bacterial basis of marine biofouling." Victoria, British Columbia, May 26, 2015.