2014-2017 NRAC FINAL PROGRESS REPORT

Project Title	13-10 New Tools to Prevent Bacterial Diseases in Shellfish Hatcheries
Reporting Period	August 2014 – September 2017
Author (Chair)	Dr. David Rowley, University of Rhode Island
Key Words	Probiotic, vibrio, shellfish, hatchery
Funding Level	Total funds allocated for this project to date. *NOTE: This could be reported by Year. i.e.,* *Year One: FY 20xx, $$ amount* *Year Two: FY 20xx, $$ amount*
Participants	*List participating personnel and respective institutions/agency/business; include outreach representative. Indicate funded participants with an asterisk.* **Name:** David C. Rowley* Role: PI Institution/Agency/Business: University of Rhode Island Address: 7 Greenhouse Road, Kingston, RI 02881 Ph: 401-874-9228 Email: drowley@uri.edu Funded: Yes ☒ No☐
Participants	**Name:** Marta Gomez-Chiarri* Role: Co-PI Institution/Agency/Business: University of Rhode Island Address: 169 CBLS, 120 Flagg Rd, Kingston, RI 02881 Ph: 401-874-2917 Email: gomezchi@uri.edu Funded: Yes ☒ No☐
Participants	**Name:** Roxanna Smolowitz* Role: Co-PI Institution/Agency/Business: Roger Williams University Address: 1 Old Ferry Rd, Bristol, RI 02809 Ph: 401-254-3299 Email: rsmolowitz@rwu.edu Funded: Yes ☒ No☐
Participants	**Name:** Dale Leavitt* Role: Co-PI Institution/Agency/Business: Roger Williams University Address: 1 Old Ferry Rd, Bristol, RI 02809 Ph: 401-450-2581 Email: dleavitt@rwu.edu

	Funded: Yes ☒ No☐
Participants	**Name:** Paul Rawson* Role: Co-PI Institution/Agency/Business: University of Maine Address: 220 Murray Hall, U Maine, Orono, ME 04469 Ph: Email: prawson@maine.edu Funded: Yes ☒ No☐
Participants	**Name:** Michael G. Devin* Role: Co-PI Institution/Agency/Business: University of Maine Address: 195 Clarks Cove Rd, Walpole, ME 04573 Ph: 207-563-3146 (x289) Email: mdevin@maine.edu Funded: Yes ☒ No☐
Project Objectives	*List each objective. (Use objectives listed in the proposal)* **Objective #1:** Formulate marine probiotic strains to create safe, effective, and stable products for use in hatchery larviculture. **Objective #2:** Conduct pilot-scale trials to test the safety and efficacy of probiotic delivery at hatcheries. **Objective #3:** Outreach and Extension: Disseminate project results to potential end users through workshops and meetings with commercial hatchery managers.
Anticipated Benefits	*State briefly how the project will benefit the aquaculture industry – directly or indirectly.* No probiotic agents are currently commercially available specifically for shellfish aquaculture. Due to successful outcomes of pilot-scale trials, our candidate probiotic strains have further advance toward commercial development as new tools to prevent bacterial diseases in shellfish aquaculture. We anticipate that these strains will ultimately be useful to prevent economic losses due to infectious disease outbreaks in commercial hatcheries. By improving upon the success of commercial hatcheries, either through improved growth and survival and/or shorter duration of the hatchery production cycle, commercial hatcheries may be able to increase overall production resulting in more seed available to growers at a reduced cost. Even if lower costs are not part of the benefit, the potential for increased seed production is critical to the further development of shellfish aquaculture in the region, as limited shellfish seed availability has already had an impact on shellfish production in the NRAC region over the past few years.
Project Progress	*Summarize concisely for each objective the progress toward accomplishment to*

	date. This has an 8,000 character limit. **Summary:** We developed several formulations of probiotic *Bacillus pumilus* RI06-95. As part of our outreach efforts, we established a relationship with a probiotic production company, Envera, to produce a potentially successful product for commercial shellfish hatcheries. We also strengthened relationships with commercial and research hatcheries to aid in testing these products. Our initial efforts were focused on the creation of stable and effective formulations of our probiotic bacteria (Objective 1). These included granulated, lyophilized, and spray-dried formulations of *Bacillus pumilus* RI06-95. Beginning in year two, our attention shifted to the testing of formulations in shellfish hatcheries (Objective 2). Hatchery experiments were conducted at Roger William University (RWU), Bristol, RI. The RWU hatchery maintains twelve 100 L conical larval rearing tanks fed from Narragansett Bay, RI, and a microalgae production greenhouse to supply daily feedings. To reduce the microbial load, raw seawater is filtered and disinfected/treated with UV light before it enters the facility. We performed five independent trials, testing each of the formulations at least once. Each trial was initiated by adding 8 -10 larvae/mL (800,000 to 1,000,000 initial larvae) per tank 1-2 days post-fertilization. Probiotic formulations were added daily at the time of feeding. Of the three formulations tested (granulated, lyophilized, spray dried), the commercially prepared spray dried (RI-SD) formulation was found to maintain the highest concentration of viable bacteria when stored at room temperature while also showing no negative impact on larval oysters in the laboratory or in the hatchery trials. After 16 weeks at room temperature, the SD-product still contained $>2.65 \times 10^{10}$ CFU/g. Our previous research has shown that probiotic concentrations of *Bacillus* products at around 1×10^4 CFU/ml provide optimal performance, meaning to reach a final target concentration of 1×10^4 CFU/ml in a 1,000 L commercial tank, only ~0.4 g of RI-695 would need to be added. This would be extremely cost effective for use at a larger scale. Another added benefit of the formulation is its ease of use. The powder quickly suspends in seawater for easy application. The spray-dried formulation was also shown to perform as well or better than freshly prepared *B. pumilus* RI06-95 in both laboratory experiments and hatchery trials. Figure 1 shows protective effects for oyster larvae challenged with the shellfish pathogen *Vibrio coralliilyticus* RE22.

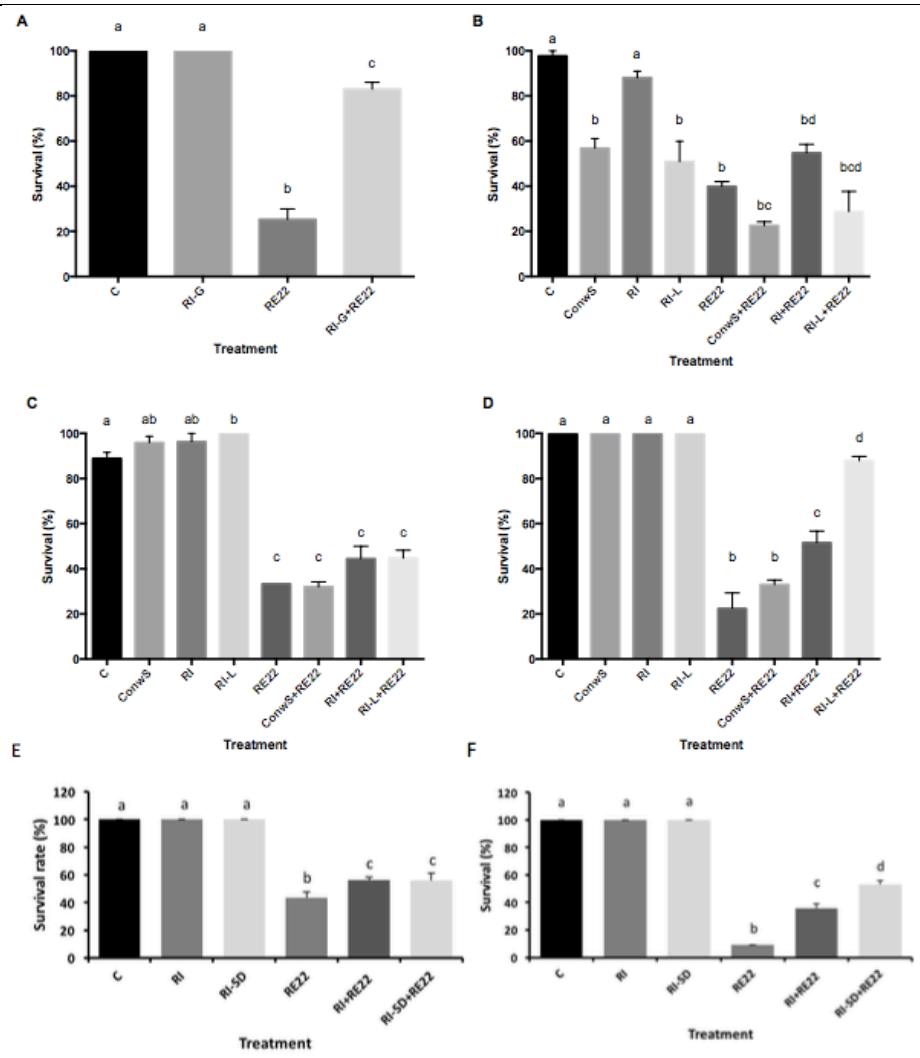

Figure 1. Laboratory challenged experiments results: Effect of pre-incubation of oyster larvae with *Bacillus pumilus* RI06-95 formulated products for 24 h on survival (% ± SEM) after challenge with *V. coralliilyticus* RE22. Survival was measured 24 h after challenge and 48 h after addition of the probiotic. (A) Exposure to a granulated product of *Bacillus pumilus* RI06-95; (B), (C), and (D) Exposure to lyophilized formulations (representative experiments) (E) and (F) Exposure to spray dried formulations. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated formulation; RI-L = lyophilized (in 100 mM sucrose) formulation 5; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. Different letters indicate statistically significant differences between the treatments.

In hatchery experiments, RI-SD showed no significant reduction in larval growth (Figure 2) or larval survival (Figure 3). In fact, it increased survival compared to freshly prepared culture in the hatchery trial by day 12. RI-SD also performed well in pathogen challenge experiments, increasing survival of larvae after the challenge at the same rate or greater as compared to freshly prepared culture (Figure 4).

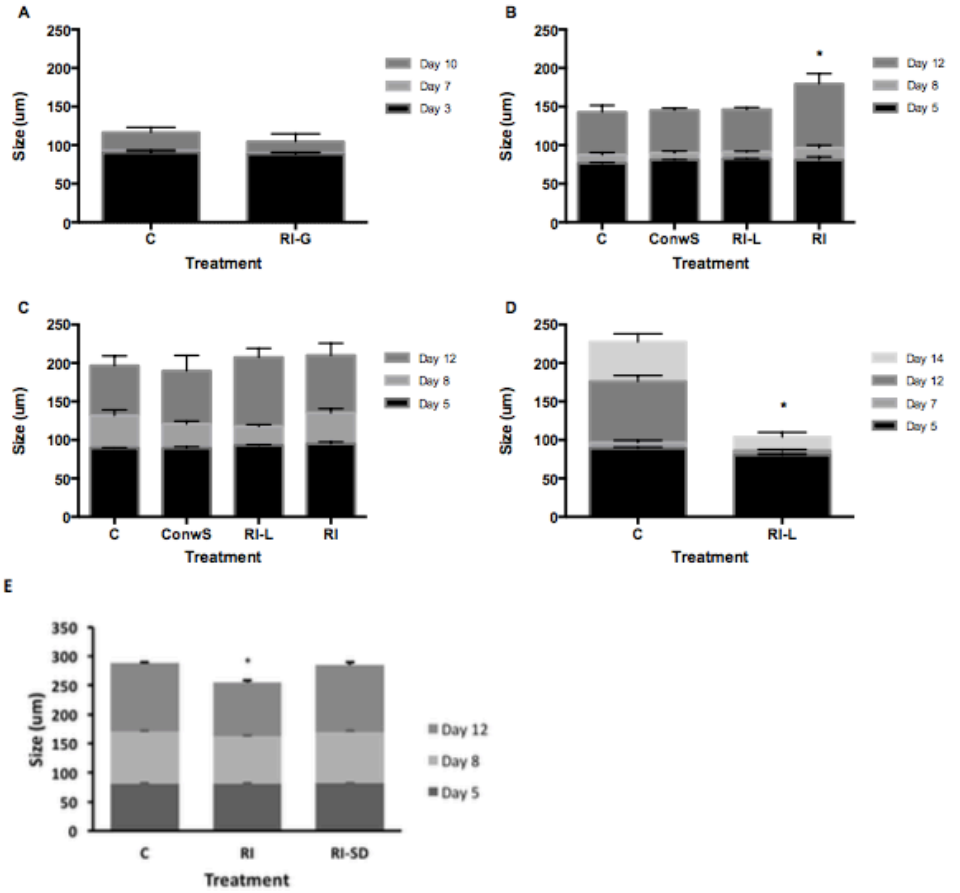

Figure 2. Effect of daily treatment with different formulations of *Bacillus pumilus* RI06-95 of larval eastern oysters (*Crassostrea virginica*) in the hatchery on mean larval size (µm ± SEM) at selected time points. (A) Trial I; (B) Trial II; (C) Trial III; (D) Trial IV and (E) Trial V. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated formulation; RI-L = lyophilized (in 100 mM sucrose) formulation; RI-SD = formulation RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. An asterisk (*) indicates statistical significances compared to controls.

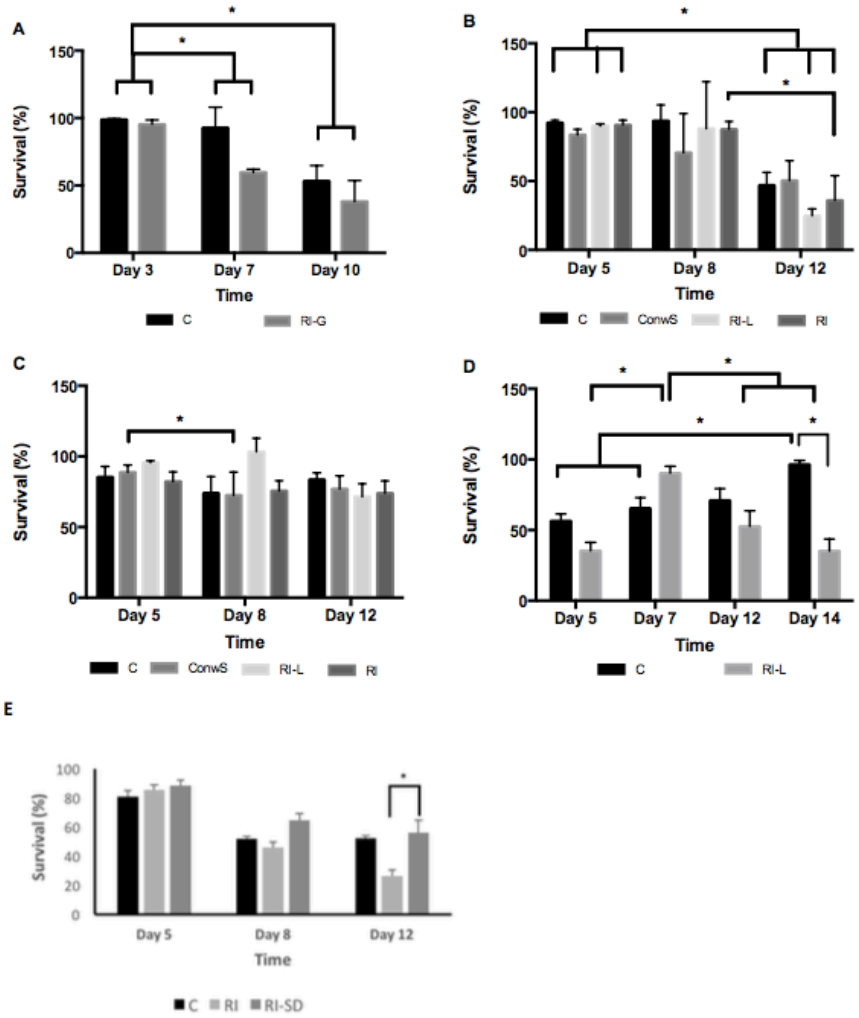

Figure 3. Effect of daily treatment with probiotics in the hatchery on interval survival (% ± SEM) of oyster larvae between selected time points. (A) Trial I; (B) Trial II; (C) Trial III; (D) Trial IV and (E) Trial V. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated formulation; RI-L = lyophilized (in 100 mM sucrose) formulation; RI-SD = spray dried formulation; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. An asterisk (*) indicates statistical significances between treatments.

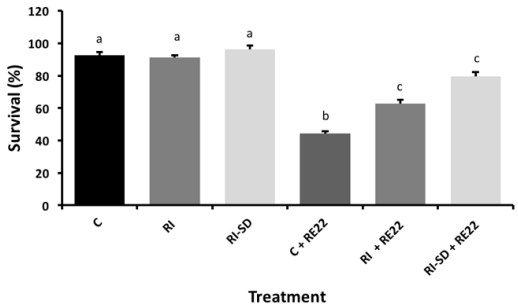

Figure 4. Effect of daily probiotic treatment in the hatchery on larval survival to a laboratory challenge with the pathogen *Vibrio coralliilyticus* RE22. Larvae were

	brought to the laboratory and survival was measured 24 h after challenge with RE22. (A) Larvae collected on Day 8 in Trial V. Abbreviations: C = no probiotic; RI-SD = spray-dried formulation; RI = freshly cultured RI06-95; RE22 = *V. coralliilyticus* RE22. A different letter indicates a significant difference between treatments (One-way ANOVA; $p < 0.05$). Conclusions. Our results demonstrate a successful spray-dried formulation of the candidate probiotic *B. pumilus* RI06-95 for its use in shellfish hatcheries. The spray dried formulation was superior to the granulated and lyophilized formulations in all criteria (stability, safety, protection). This project demonstrates the challenge in formulating a probiotic and the need for thorough testing in both laboratory and hatchery settings to confirm the desired effect. The laboratory and hatchery trials confirm that the RI-SD formulation is stable over a long term, remains viable and shows comparable performance to freshly grown cultures of the probiotic. It is suitable for storage, transportation and can be easily applied in a hatchery by mixing with seawater.
Accomplishments:	
Outreach Overview	Describe in general how your results have been extended to the intended users. OR, if they haven't yet, explain when & how this will occur. Our results indicate that our probiotics can be used to manage disease in shellfish hatcheries, since: a) these probiotics can be formulated for easy delivery in hatcheries; and b) the probiotics are effective against diseases affecting different bivalve species (hard clams, oysters, razor clams, bay scallops), as demonstrated in laboratory and hatchery scale experiments. The results from this research have been presented at several **meetings**, two regional (the Milford Aquaculture Seminar, Mystic, Connecticut, February 2014; and the Northeast Aquaculture Conference and Exposition, Portland, Maine, January 2015) and three national (Annual Meeting of the National Shellfisheries Association, Jacksonville, Florida, March 2014 and Monterey, California, March 2015; Eastern Fish Health Workshop, Charleston, SC, April 2015). These meetings are widely attended by the shellfish industry and researchers in bivalve health. At these meetings, we presented results from our research with probiotics, as well as described how probiotics can be integrated with other disease management tools, such as the use of disease resistant strains or monitoring water quality. Hatchery managers were very excited about the results from our research. Another component of our outreach efforts was reaching out to companies involved in commercialization of products for aquatic animal health management. Finally, and as a result of the presentation at the National Shellfisheries Association meeting, we established an **international collaboration** with a group in Spain (led by Dr. Juan Barja, Universidad de Santiago de Compostela) that has an established relationship with many shellfish hatcheries in Spain. As a result of this collaboration, we hosted a Spanish student in our laboratories during the Fall of 2014 (resulting in a publication submitted to Journal of Invertebrate Pathology) and expanded our studies to pathogens and probiotics isolated from hatcheries in Spain.
Targeted Audiences	Provide information on the **target audience** for efforts designed to **cause a change in knowledge, actions, or conditions**.

	The main target audience for this project is **research and commercial shellfish hatchery managers**. The goal of our research and outreach efforts is to provide hatchery managers with environmentally friendly, economic, and effective tools to manage infectious diseases in shellfish hatcheries. Our research and outreach efforts also target **aquatic pathologists and microbiologists**, by providing knowledge about potential mechanisms of action of these probiotics, knowledge that is fundamental for the rational development of effective and safe probiotics. We have also targeted **aquatic animal health companies** that are interested in the commercialization and distribution of our probiotics, and established relationships with a probiotics company (Envera, West Chester, PA) for the commercialization of our product.
Outputs	Outputs are tangible, measurable products (website, events, workshops, products [AV, curricula, models, software, technology, methods, websites, patents, etc.], trainees, etc.). Do NOT include publications as they're listed separately. Outputs from this research include presentations at scientific conferences, abstracts published in the Journal of Shellfish Research, and training of 4 graduate students and 7 undergraduate students. This research will lead to 4 Ph.D. dissertations. In addition, we have established a Materials Transfer Agreement with a company, Envera that produces probiotics at a commercial scale. A successful formulation has been crafted and looks promising for translation into a commercial product.
Outcomes/Impacts	Describe how findings, results, techniques, or other products that were developed or extended from the project generated or contributed to an outcome/impact. **Outcomes/impacts are defined as changes in Knowledge, Action, or Condition.** The general outcome of this research is the development of an environmentally friendly, economic, and effective method for the management of infectious diseases in bivalve shellfish hatcheries. This method will serve as an alternative to antibiotic treatments (not allowed by FDA regulations) and the need for expensive water treatment systems.
Impacts Summary	Provide short statements (2-3 sentences) about each of the following: (pre-established fields for Researchers to complete short statement answers) 1. **Relevance:** Issue – what was the problem? Pathogenic bacteria, especially those belonging to the genus *Vibrio*, cause devastating disease outbreaks in shellfish larviculture that result in substantial financial losses for commercial hatcheries. 2. **Response:** What was done? We previously discovered several probiotic strains that promote survival of oyster larvae when challenged with pathogens. We have prepared probiotic formulations suitable for delivery at shellfish hatcheries and

	tested their safety and efficacy. 3. **Results:** How did your work make a difference **(change in knowledge, actions, or conditions)** to the target audiences? Our work has potentially resulted in a new product to prevent bacterial infections in shellfish larvae and seed. 4. **Recap:** One- sentence summary Our formulated probiotic bacteria will help hatchery managers prevent infectious disease outbreaks during shellfish larviculture.
Publications	Follow the format to list publications in the following categories: • Presentations: o Oral Gomez-Chiarri, M., Rowley, D., Nelson, D.R., Proestou, D., Frank-Lawale, A., Allen Jr., S.K., Guo, X. and Rawson, P.D. 2014. Disease management strategies for shellfish aquaculture: The important role of hatcheries. *Journal of Shellfish Research* . Abstract of the Milford Aquaculture Seminar, Shelton, CT, February 2014. Sohn, S., Dao, C., Zhao, W., Kessner, L., Volpe, L., Rowley, D., Nelson, D.R. and Gomez-Chiarri, M. 2014. Development of probiotic formulations for shellfish hatcheries. *Journal of Shellfish Research* 33(2):654. 105th Annual meeting of the National Shellfisheries Association, Jacksonville, FL, March 2014. Gomez-Chiarri, M., Zhao, W., Sohn, S., Dao, C.A., Rowley, D., Nelson, D.R., 2015. Fight them using their own tools (and some others): The role of biofilm formation on the probiotic activity of Phaeobacter gallaeciensis S4. Presented at the Eastern Fish Health Workshop, Charleston, SC, March 2015. Sohn, S.B. Rowley, D., Nelson, D.R., Smolowitz, R.M, Gomez-Chiarri, M. 2015. The effects of candidate probiotics on several species of cultured larval shellfish. Proceedings of the Northeast Aquaculture Conference and Exposition, Portland, Maine, January 2015. Sohn, S.B., Zhao, W., Rowley, D., Nelson, D.R., Smolowitz, R.M., Gomez-Chiarri, M., 2015. Probiotics for shellfish hatcheries: From mechanisms of action to hatchery trials. *J. Shellfish Res*. Abstracts of the Annual Meeting. Presented at the National Shellfisheries Association, Journal of Shellfish Research, Monterey, CA, March 2015. o Posters 2 • Peer-reviewed: o Print – *in preparation for J Shellfish Research* Title: Use of formulated probiotic *Bacillus pumilus* RI06-95 for preventing Vibriosis in larviculture of the Eastern oyster *Crassostrea virginica* Authors: Saebom Sohn[1], Tejashree Modak[1], Victor Schmidt[1], Christine Dao[2], Meagan Hamblin[2], Marta Gómez-Chiarri[1], David R. Worthen[2], Kathryn Markey

	Lundgren[3], Karin Tammi[3], Roxanna Smolowitz[3], Lauren Gregg[4], Standish K. Allen Jr. [4], David R. Nelson[5], and David C. Rowley[2,*] o Digital (websites, videos, etc.) 0 • Non-Peer-reviewed: o Extension factsheets o Popular articles 0
Students/Participants (URI)	Provide the following information for **every** student that worked with you during the reporting period: • Name: Christine Dao • Whether Degree was completed during the reporting period (name, yes/no): Yes • New or Continuing Student: Continuing • Capstone/Thesis Title (actual or anticipated): Chemical investigation of candidate probiotics in aquaculture and formulation of a probiotic agent for oyster aquaculture. • Date of Graduation: PhD, Spring 2015
Students/Participants (URI)	• Name: Sae Bom Sohn • Whether Degree was completed during the reporting period (name, yes/no): No • New or Continuing Student: Continuing • Capstone/Thesis Title (actual or anticipated): Evaluation of the efficacy of candidate probiotics for disease prevention in shellfish hatcheries • Date of Graduation: PhD, Fall 2015
Students/Participants (URI)	• Name: Meagan Hamblin • Whether Degree was completed during the reporting period (name, yes/no): Yes • New or Continuing Student: Continuing • Date of Graduation: BS Pharmaceutical Sciences, May 2016
Students/Participants (URI)	• Name: Thomas Rylah • Whether Degree was completed during the reporting period (name, yes/no): No • New or Continuing Student: Continuing • Date of Graduation: BS, May 2017
Students/Participants (URI)	• Name: Tejashree Modak • Whether Degree was completed during the reporting period (name, yes/no): No • New or Continuing Student: New

<table>
<tr><td></td><td colspan="4">• Date of Graduation: PhD, May 2018</td></tr>
<tr><td>Students/Participants
(URI)</td><td colspan="4">• Name: Hilary Ranson
• Whether Degree was completed during the reporting period (name, yes/no): No
• New or Continuing Student: New
• Date of Graduation: PhD, May 2020 (anticipated)</td></tr>
<tr><td>Students/Participants
(RWU)</td><td colspan="4">• Name: Caroline Call
• Whether Degree was completed during the reporting period (name, yes/no): BS, yes
• New or Continuing Student: New
• Date of Graduation: May 2014</td></tr>
<tr><td>Students/Participants
(RWU)</td><td colspan="4">• Name: Nathan Canfield
• Whether Degree was completed during the reporting period (name, yes/no): BS, no
• New or Continuing Student: Continuing</td></tr>
<tr><td>Students/Participants
(RWU)</td><td colspan="4">• Name: Molly Waters
• Whether Degree was completed during the reporting period (name, yes/no): BS, yes
• New or Continuing Student: Continuing
• Date of Graduation: May 2015</td></tr>
<tr><td>Students/Participants
(RWU)</td><td colspan="4">• Name: Jose Garcia
• Whether Degree was completed during the reporting period (name, yes/no): BS, yes
• New or Continuing Student: Continuing
• Date of Graduation: May 2015</td></tr>
<tr><td>Students/Participants
(RWU)</td><td colspan="4">• Name: Elizabeth McGarvey
• Whether Degree was completed during the reporting period (name, yes/no): BS, yes
• New or Continuing Student: New
• Date of Graduation: May 2015</td></tr>
<tr><td rowspan="2">Partnerships</td><td colspan="4">List any partners that you worked with on your project. Provide the following information for each Partner:</td></tr>
<tr><td>Partner</td><td>Specific Type
Type</td><td>Level
Level</td><td>Nature of Partnership</td></tr>
</table>