ANNUAL REPORT

NORTHEASTERN REGIONAL AQUACULTURE CENTER

For the Period
September 1, 2016 to August 30, 2017

United States Department of Agriculture
National Institute of Food and Agriculture

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Northeastern Regional Aquaculture Center

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Introduction

The Northeastern Regional Aquaculture Center is one of five Regional Aquaculture Centers established by the U.S. Congress under the National Aquaculture Research, Extension and Teaching Policy Act of 1977 (Subtitle L, Section 1475(d)) and subsequent authorized legislation. These centers, located in the northeast, southern, north central, western, and the topical/sub-tropical Pacific regions are administered by the U.S. Department of Agriculture, National Institute of Food and Agriculture (NIFA). Located at universities and/or research institutions the regional centers' mission is to support aquaculture research, development, demonstration, and extension education to enhance viable and profitable U.S. aquaculture production which will benefit consumers, producers, service industries, and the American economy.

Organization and Administration

Regional Centers

The Regional Aquaculture Centers are administrative rather than physical centers. The Centers provide a means of assessing research and extension needs, assuring industry input, establishing priorities, and implementing aquaculture research and extension programs. The Centers facilitate implementation, administration, and coordination of regional research and extension programs, and they foster information exchange, research and extension linkages, and cross fertilization of ideas within and between regions and between organizations.

Organization

The Northeastern Regional Aquaculture Center (NRAC) has an administrative staff consisting of a one half-time Director, an Administrative Assistant, and a Coordinator. NRAC's Board of Directors (BOD) is the policy making body for NRAC and consists of nine members representing the Dean of the College of Agriculture and Natural Resource at the University of Maryland Regional Agriculture Experiment Directors, the Regional Extension Directors, the 1890 Schools, Agricultural Research Service laboratories in the Northeast Region, Sea Grant Directors, and industry or private institutions. The BOD also has responsibility for approval of all NRAC projects. The BOD is assisted by an Industry (IAC) and a Technical (TAC) Advisory Committee. The IAC, with assistance from the TAC, summarizes industry research and extension priorities for the Northeastern regional aquaculture industry and assists in assuring these priorities are incorporated into NRAC planning. The TAC, with help from the IAC, assists NRAC in assuring high quality projects having good science and addressing industry priorities are funded by NRAC.

The IAC and TAC are both comprised of one representative from each of the 12 states in the Northeastern Region and the District of Columbia. These states include Connecticut, Delaware, the District of Columbia, Maine, Maryland, Massachusetts, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, and West Virginia. Thus, there are 13 members on each committee who provide representation from all parts of the region and for the various sectors of the aquaculture industry. The TAC is divided into representatives from the research and the extension communities who provide their expertise to NRAC in defining priorities and selecting high quality research and extension projects.

Administrative Operations

The Northeastern Regional Aquaculture Center was located at the University of Massachusetts Dartmouth from 1988 until 2004. At that time the University of Massachusetts Dartmouth decided their priorities had changed and no longer wished to host NRAC. Through a competitive process the University of Maryland was selected by USDA, CSREES (Cooperative States Research, Education and Extension Service) to host NRAC and in December of 2005 NRAC was transferred to the University of Maryland at College Park, Maryland. The University of Massachusetts and the University of Maryland worked to complete transfer of NRAC to the University of Maryland. Because of constraints on funding FY 2006 funds were the first funds coming directly from USDA to the University of Maryland. Projects are complete and NRAC funds held at the University of Massachusetts Dartmouth have been expended, the University of Maryland has become responsible for all of NRAC activities and the University of Massachusetts has been phased out of NRAC activities. The completion of all involvement in NRAC by the University of Massachusetts Dartmouth ended in September 30, 2010.

All NRAC staff members are at the University of Maryland and the day to day operations of NRAC are operating out of the University of Maryland. The NRAC Director reports to the Dean of the College of Agriculture and Natural Resources, University of Maryland at College Park. Dr. Fredrick W. Wheaton, who was instrumental in moving the Center from UMASS Dartmouth, retired as the NRAC Director on June 30, 2010. He was replaced by Dr. Reginal M. Harrell effective July 1, 2011.

Board of Directors

The BOD members serve four-year terms except for some of the initial BOD members who will have shorter terms to develop the staggered terms needed to provide continuity over time. The Sea Grant Director serves a two-year term. The current BOD members are:

Board Member	Representing	State Where Located	Term Ending
Dr. Adel Shirmohammadi	Associate Dean/Associate Director, MD Agricultural Experiment Station University of Maryland	Maryland	Permanent Seat
Dr. Moses T. Kairo	1890 Land Grant Colleges	Delaware	December 2018
Dr. Richard Rhodes (Board Chair)	Experiment Station Director	Rhode Island	December 2016
Dr. Caird Rexroad	ARS	Maryland	December 2018
Dr. Fredrika C. Moser	Sea Grant Director	Maryland	December 2016
Dr. Michael O'Neill	Extension Director	Connecticut	December 2017
Dr. Ferderick A. Servello	Experiment Station Director	New Jersey	December 2018
Karl R. Roscher	Industry	Maryland	December 2018
Dr. William Hare	Extension Director	District of Columbia	December 2019

Industry Advisory Committee
Composition, Appointment, and terms of IAC

The IAC is comprised of representatives from the District of Columbia and the 12 states in the Northeast Region. They serve three-year terms except for the first IAC, which will have varying length appointments to develop the staggered terms needed to provide continuity for the IAC. Current members of IAC are:

IAC Member	Organization	State
Vacant		Connecticut
Mr. John W. Ewart	Delaware Sea Grant	Delaware
Mr. Matthew E. Moretti	Wild Ocean Aquaculture, LLC	Maine
Mr. Talmage Petty	Hollywood Oysters	Maryland
Dr. Daniel Ward (Co-chair)	Ward Aquafarms	Massachusetts
Vacant	Parsons Seafood	New Jersey
Mr. Brian S. Gennaco	Virgin Oyster Company, LLC	New Hampshire
Dr. Steve Malinowski	Fishers Island Oyster Farm	New York
Vacant		Pennsylvania
Vacant		Rhode Island
Vacant		Vermont
Mr. Greg Casten	ProFish	Washington, DC
Mr. Daniel Miller (Chair)	Potesta & Associates, Inc.	West Virginia

Composition, Appointment, and terms of TAC

The TAC is comprised of representatives from the District of Columbia and the 12 states in the Northeast Region. They serve three year terms except for the first TAC which will have varying appointments to develop the staggered terms needed to provide continuity for the TAC. The TAC is divided into two groups with approximately one-half of the members representing research and approximately one-half representing extension. Current members of TAC are:

TAC Member	**State**	**Extension/ Research**
Vacant	Connecticut	
Dr. Dennis McIntosh	Delaware State University	Extension
Dr. Stephen D. Eddy	Center for Cooperative Aquaculture Research, Maine	Research
Mr. Don Webster	University of Maryland	Extension
Vacant	Massachusetts	
Dr. Elizabeth Fairchild	University of New Hampshire	Research
Dr. Daphne Munroe (Chair)	Haskin Shellfish Research Laboratory, New Jersey	Research
Vacant	Pennsylvania	Extension
Dr. Rodman Getchell	Cornell University	Research
Dr. Marta-Gomez-Chiarri (Co-chair)	University of Rhode Island	Research
Dr. Matthew L. Richardson	Univ of the District of Columbia	Extension
Vacant	Vermont	
Dr. Patricia M. Mazik	West Virginia University	Research

Project Development

NRAC has two methods to develop projects: 1) the RFA method and 2) the project group method. The IAC develops priorities and the TIAC (Technical and Industrial Advisory Council comprised of the IAC and TAC together) develop problem statements to convert the priorities into researchable statements. The problem statements are distributed through the Northeast Region with a RFA (Request for Applications). Thus, anyone interested in submitting a proposal may submit a proposal as long as it addresses the problem statements. In some situations there will be a pre-proposal stage and then only selected (by the TIAC) pre-proposals will be invited to submit full proposals. The RFA method is the most common method used by NRAC. The group project is a process where a priority is defined, a problem statement is prepared and a request for a statement of interest is distributed throughout the Northeast region. People responding to the statement of interest are then brought together to develop a proposal to address the problem statement. The project group method tends to work well in some situations such for extension projects. Currently the Northeast Regional Extension project is the only project for which NRAC has used the project group method.

Projects 2016-2017

NRAC's program year runs from September 1 to August 30 annually. This report covers the 2017 program year (September 2016 through August 2017). During this period NRAC has provided funding to eight research and extension projects in addition to administrative projects. In the last 12 months NRAC has committed over $500,000 to projects and NRAC operations. Completion or project progress reports are included in this document for projects that have been in existence long enough to have submitted a progress or final reports.

Table 1 lists the projects by title and total project funding level. Details of the projects including project titles, abstracts, total funding, project numbers, and project results and findings to date are available in the appendix of this report. Publications, videos, extension publications, and other written or visual materials produced as part of each project are listed to the extent available for each project. Although attempts were made to be as complete as possible some publications that resulted from NRAC funding, particularly papers presented and papers published in peer reviewed literature, may not be included due to the time lag between the end of a project and the publication of results.

Table 1. NRAC projects active during 2016-2017

NRAC Project Title	Total Budget	Start Date	End Date
Safe Feedstock for Bivalve Aquaculture	$200,000	9/1/15	9/30/2017
Develop of Novel, Nontoxic Solutions for Biofouling Control and Predator Exclusion in Shellfish Aquaculture	$193,582	9/1/15	9/30/2017
Testing and Application of Novel Probiotic Bacteria for Use in Marine Aquaculture	$190,508	9/1/15	9/30/2017
Genetic Marker-assisted selection of Northeastern hard clams for QPX resistance	$199,998	11/1/2012	1/31/2017

New Tools to Prevent Bacterial Disease in Shellfish Hatcheries	$199,514	10/1/2013	9/30/2017
White Worm *Enchytraeu albidus*, Production and Marketing for Live Aquaculture Feed	$144,677	8/15/14	8/3120/17
Improved Grow-out Methodologies for Razor Clams	$176,049	7/1/13	8/31/2017
Development and evaluation of novel, non-toxic solutions for biofouling control and predator exclusion in shellfish aquaculture	$193,582	10/01/2016	9/30/2018

Accomplishments

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED:

Peer-reviewed publications:

Wang K, Del Castillo C, Corre E, Pales Espinosa E, Allam B. (2016). Clam focal and systemic immune responses to QPX by RNA-Seq technology. BMC Genomics 17:146.

Wang K, Pales Espinosa E, Tanguy A, Allam B. (2016). Alterations of the immune transcriptome in resistant and susceptible hard clams (Mercenaria mercenaria) in response to Quahog Parasite Unknown (QPX) and temperature. Fish and Shellfish Immunology 49: 163-176.

Allam B, Pales Espinosa E. (2016). Bivalve immunity and response to infections: Are we looking at the right place? Fish and Shellfish Immunology, 53: 4-12

Dahl S, Allam B. (2016). Hard clam relocation as a potential strategy for QPX disease mitigation within an enzootic estuary. Aquaculture Research 47(11):3445-3454.

Allam B, Raftos D. (2015). Immune responses to infections. Journal of Invertebrate Pathology 131: 121-136.

Fairchild, E. A., A. M. Bergman, and J. T. Trushenski. 2017. Production and nutritional composition of white worms *Enchytraeus albidus* fed different low-cost feeds. Aquaculture 481: 16-24.

Extension factsheets:

Fairchild, E. A. and M. L. Walsh. 2017. How to grow white worms. NRAC Fact Sheet No. 223-2017.

Fairchild, E. A., M. L. Walsh, J. T. Trushenski, K. L. Cullen, and M. Chambers. 2017. White worms – a low cost live feed for the ornamental industry. NRAC Fact Sheet No. 224-2017.

In Preparation:

Guo X, Wang G, Pales Espinosa E, del Castillo C, Tanguy A, Kraeuter J, Allam B. Identification of QPX-resistance markers by genomewide candidate-gene association study in the hard clam.

Oral Presentations:

Allam B, Pales Espinosa E, Wang G, Smolowitz R, Murphy D, Rivara G, Guo X. (2017). Development of strategies to mitigate QPX disease in the hard clam. Northeastern Aquaculture Conference and Expo. January 11-13, 2017.

Providence, Rhode Island, USA.

Guo X, Wang G, del Castillo C, Pales Espinosa E, Tanguy A, Kraeuter J, Allam B (2016). Identification of QPX-resistance markers by genome-wide candidategene association study in the hard clam. World Aquaculture Society Triennial Meeting, Las Vegas, NV. February 22-26, 2016.

Fairchild, E. A. and J. T. Trushenski. 2018. Improving white worm *Enchytraeus albidus* nutrition for ornamental fishes. Ornamental Fish Session. The annual meeting of the World Aquaculture Society, February 19-22, 2018, Las Vegas, NV. (invited talk; accepted presentation)

Fairchild, E. A., M. Chambers, and M. L. Walsh. 2017. Do white worms have commercial potential as a feed in the ornamental industry? Ornamental Fish Session. The annual meeting of the World Aquaculture Society, February 20-22, 2017, San Antonio, TX.

Bergman, A., J. T. Trushesnki, and E. A. Fairchild. 2016. Cultivation of white worms *Enchytraeus albidus* using low- or no-cost feed resources. Aquaculture 2016. The annual meeting of the World Aquaculture Society, February 22-26, 2016, Las Vegas, NV.

Fairchild, E. A. and E. Groover. 2016. Effects of feeds and temporal cycles on white worm *Enchytraeus albidus* production. Aquaculture 2016. The annual meeting of the World Aquaculture Society, February 22-26, 2016, Las Vegas, NV.

Fairchild, E. A. 2015. Aquaculture initiatives at the Coastal Marine Lab. University of New Hampshire Department of Biological Sciences Sustainable Agriculture Seminar Series, September 18, 2015, Durham, NH.

Smalls, J. and D. McIntosh. The Use of Probiotics in Shrimp Aquaculture. Aquaculture America 2017 Book of Abstracts, San Antonio, TX, USA.

Myer, J. L., and D. McIntosh. Probiotics and Fish Growth. 2017 ARD Research Symposium, Atlanta, GA.

Smalls, J. and D. McIntosh. The Use of Probiotics in Shrimp Aquaculture. 2017 ARD Research Symposium, Atlanta, GA.

Shumway, S., Walsh, Bullard and Getchis. 2016. Biofouling Workshop for Industry. NACE Meeting Providence, R.I. December 2016

Shumway, S. 2017. The National Shellfisheries Association Annual Conference. Knoxville, TN

Shumway, S. 2017. US Aquaculture Meeting. San Antonio, TX, February 2017

Shumway, S. 2017. International Pectinid Workshop. Portland, ME, April 2017

Shumway, S. 2016. Ocean University Shanghai, China. May 2016

Shumway, S. 2016. FENAOSTRA (National Oyster Fair), an industry exposition in Florianopolis, Brazil. September 2016

Shumway, S. Erasmus Mundas Graduate Training Program in Aquaculture, University of Nantes, France. November 2016

Shumway, S. City University of Hong Kong, Hong Kong, China. May 2017

Shumway, S. The Kenneth K. Chew Center for Research and Restoration, NOAA Northwest Fisheries Science Center Seattle, Washington. July 2017.

Shumway, S. University of Maine at Machias. August 2017

Posters:

Kailai Wang (2016). Molecular characterization of clam (Mercenaria mercenaria) immune responses against Quahog Parasite Unknown (QPX): Effect of host and environmental factors. http://gradworks.umi.com/10/13/10139857.html

Fairchild, E. A. and C. Giray. 2016. White worms *Enchytraeus albidus*: a pathogen-free live feed? Aquaculture 2016. The annual meeting of the World Aquaculture Society, February 22-26, 2016, Las Vegas, NV.

Myer, J. L., E. Schott, H. J. Schreier, and D. McIntosh. Probiotics and Fish Growth. Aquaculture America 2017 Book of Abstracts, San Antonio, TX, USA

APPENDICES

A - Final Reports

B - Progress Reports

APPENDIX A

PROJECT COMPLETION REPORT

Genetic Marker-Assisted Selection of Northeastern Hard Clams for QPX-Resistance
Subaward # Z555103
Grant # 2012-38500-19656

PROJECT CODE: **SUBCONTRACT/ACCOUNT:**

PROJECT TITLE: Genetic Marker-Assisted Selection of Northeastern Hard Clams for QPX-Resistance

DATES OF WORK: 02/01/2013-01/31/2017 (including 1 year no-cost extension)

FUNDING LEVEL: $199,998

PARTICIPANTS:

Principal Investigators:

Bassem Allam / Professor / School of Marine and Atmospheric Sciences / Stony Brook University / Stony Brook, NY 11794-5000 / Phone: 1 631 632 8745 / Fax: 1 631 632 8915 / E-mail: bassem.allam@stonybrook.edu - Funded

Ximing Guo / Professor / Haskin Shellfish Research Laboratory / Rutgers University / 6959 Miller Avenue / Port Norris, NJ 08349 / Phone: (856) 785-0074 ext. 4324 / Fax: (856) 785-1544 / E-mail: xguo@hsrl.rutgers.edu - Funded

Roxanna Smolowitz / Associate Professor / Roger Williams University / One Old Ferry Road, Bristol, RI 02809 / Phone: (401) 254-3299 / Fax: (401) 254-3310/ Email: rsmolowitz@rwu.edu – Funded

Emmanuelle Pales Espinosa / Research Scientist / School of Marine and Atmospheric Sciences / Stony Brook University / Stony Brook, NY 11794-5000 / Phone: 1 631 632 8694 / Fax: 1 631 632 8915 / E-mail: epalesespino@notes.cc.sunysb.edu - Funded

Gregg Rivara / Aquaculture Specialist / Cornell University Cooperative Extension of Suffolk County / 3690 Cedar Beach Road / Southold, NY 11971 / Phone: (631) 852 8660 ext. 35 / Fax: (631) 852 8662 / Email: gjr3@cornell.edu - Funded

Cooperating Participants:

George (Gef) Flimlin / Marine Extension Agent / Rutgers Cooperative Extension / 1623 Whitesville Road / Toms River, NJ 08755 / Phone: (732) 349-1152 / Fax: (732) 505-8941 / Email: flimlin@aesop.rutgers.edu – Unfunded

Diane Murphy / Fisheries & Aquaculture Specialist / Cape Cod Cooperative Extension & Woods Hole Sea Grant / Box 367 / Barnstable, MA 02630 / Phone: (508) 375 6953 / Fax: (508) 362-4923/ Email: dmurphy@whoi.edu - Funded

Arnaud Tanguy / Associate Professor / University of Paris 6 / Station Biologique de Roscoff / Place Georges Teissier BP74/ 29682 Roscoff, France / Phone: +33 298 292 527 / Fax: +33 298 292 324 / Email: atanguy@sb-roscoff.fr - Unfunded

Antoinette Clemetson / Fisheries Specialist / NY Sea Grant / 3059 Sound Avenue / Riverhead, NY 11901 / Phone: (631) 727-3910 / Fax: (631) 369-5944 / Email: aoc5@cornell.edu - Unfunded

REASON FOR TERMINATION: Objectives completed and funds terminated

PROJECT OBJECTIVES:

- **Objective 1:** Select candidate genes based on sequence information generated from our prior investigations and validate single nucleotide polymorphism loci for clam genotyping
- **Objective 2:** Proof-test the link between the polymorphism of the candidate genes and QPX resistance on samples preserved from prior field work preceding and following QPX-related clam mortalities
- **Objective 3:** Validate the markers identified in Objectives 1 and 2 for the assessment of the resistance of different seed strains used for aquaculture along the east coast during QPX exposure studies
- **Objective 4:** Provide the aquaculture industry with superior germlines derived from selected clams surviving QPX-related mortalities

ANTICIPATED BENEFITS:

The genetic markers identified in this project are expected to represent a useful method for forecasting clam resistance to QPX infection. A direct outcome of this research is the identification of resistant clam stocks that will help the aquaculture industry face QPX disease outbreaks. The project will also generate important genomic information that will be made public, fostering research on this economically and ecologically important species.

PRINCIPAL ACCOMPLISHMENTS:

Objective 1: Transcriptomic sequence data from previous research (707 Million Illumina 100 bp reads) were assembled and screened for single nucleotide polymorphism (SNP). Assembled transcripts included a total of 66,378 contigs displaying SNP variations, in addition to 568 and 426 contigs displaying deletion/insertion variants or multiple nucleotide variants, respectively. An additional 19,037 contigs simultaneously displayed 2 or more types of variations. All contigs were functionally annotated and a total of 384 immune-related genes that have SNPs transcripts were used to genotype clams using genotyping by sequencing approaches (Ion AmpliSeq method in conjunction with next generation sequencing). Primer pairs were successfully designed for 373 transcripts. They were synthesized and pooled for multiplex amplification and genotyping of clams sampled before and after QPX disease outbreaks.

Objective 2: Hard clams were collected from three stocks before deployment in Massachusetts and New Jersey. The deployed clams were sampled again after field mortalities (47 – 93%) caused primarily by QPX. DNA from 56 - 64 clams were pooled in equal amounts producing 3 before and 6 after-mortality DNA pools. The pooled DNAs were used as templates for amplification of 373 candidate genes with the AmpliSeq primer panel. Amplified products were sequenced to about 1000x per gene with the Ion Torrent PGM 400 bp module. Of the 373 genes targeted, 98 genes were successfully amplified and sequenced in all 9 samples. SNPs and indels were identified and analyzed for post-mortality frequency shifts. Nine SNPs in seven genes showed consistent allele frequency shifts in all three stocks and at both sites, suggesting they may be linked to QPX-resistance or survival.

Objective 3: A total of 5 clam strains were deployed in a field site in NY in early July 2014 including 3 custom-spawned clams (SC, NJ and MA strains derived from clams that survived QPX mortalities) and 2 commercial strains (NY and a second MA strain hereby designated MA2) (5 replicates each). The 2 commercial clam strains (NY and MA2) were also deployed in MA. Deployed clams were monitored for 2 years and results showed marked difference in clam resistance to QPX disease in the MA site (disease prevalence in NY was low for all strains). Interestingly, results showed that the disease developed significantly more in the MA clam strain as compared to the NY strain (obtained from Frank M. Flowers and Sons Oyster Co. Oyster Bay, NY) with prevalence averaging 50% and 10%, respectively. DNA samples from clams collected before and after deployment are being submitted to genotyping to validate the genetic markers identified in Objective 2. The initial delay in the establishment of the award led to a delay in field deployment and final sample collection. We expect the validation step to be completed in the next 3 months.

Objective 4: The project allowed the identification of resistant clam stocks (Frank M. Flowers and Sons line). This clam line is available and will be evaluated for use in breeding programs throughout the Northeast. Upon validation, the new genetic markers will also be published and shared with stakeholders and scientists for use in marker-assisted selection programs.

IMPACTS:

- For the first time, genetic markers have been associated with survivorship following QPX outbreaks
- Identified clam stocks that are resistant to QPX disease
- Communicated study results to stakeholders for the promotion of resistant clam stocks
- Provided the industry (growers in MA, NY and NJ) with disease testing results on clam broodstock and seeds

RECOMMENDED FOLLOW-UP ACTIVITIES:

Study results served as a base for the development of a new research program recently funded by the USDA (NIFA) to validate the identified genetic markers as predictors for clam resistance to the infection (not just as being correlated to resistance). We propose to extend this research to the discovery of additional genetic markers linked to resistance and overall yield. Further, the potential benefits of integrating resistant broodstocks (Frank M. Flowers and Sons line) into the breeding programs of commercial hatcheries throughout the Northeast needs to be assessed.

SUPPORT:

Year	NRAC-USDA funding	Other support					Total support
		University	Industry (in-kind)	Other Federal	Other (in-kind)	Total	
1	68,502	14,900 [1]	Clams [2]	0	Field support [3]	14,900	83,402
2	63,123	14,900 [1]	0	0	Field support [3]	14,900	78,023
3	68,373	14,900 [1]	0	0	Field support [3]	14,900	83,273
Total	199,998	44,700 [1]	0	0	Field support [3]	44,700	244,698

[1] The university cost share is contributed by Stony Brook University as support to Allam's academic salary.
[2] Clams were provided by three commercial hatcheries located in MA and NJ (names of hatcheries are not presented here to maintain confidentiality) and NY (Frank M. Flowers and Sons, Oyster Bay, NY).
[3] Field support was provided by the New York State Department of Environmental Conservation (boat use + captain time) for accessing the field site in NY

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED:

- Peer-reviewed publications:

Wang K, Del Castillo C, Corre E, Pales Espinosa E, Allam B. (2016). Clam focal and systemic immune responses to QPX by RNA-Seq technology. BMC Genomics 17:146.

Wang K, Pales Espinosa E, Tanguy A, Allam B. (2016). Alterations of the immune transcriptome in resistant and susceptible hard clams (Mercenaria mercenaria) in response to Quahog Parasite Unknown (QPX) and temperature. Fish and Shellfish Immunology 49: 163-176.

Allam B, Pales Espinosa E. (2016). Bivalve immunity and response to infections: Are we looking at the right place? Fish and Shellfish Immunology, 53: 4-12

Dahl S, Allam B. (2016). Hard clam relocation as a potential strategy for QPX disease mitigation within an enzootic estuary. Aquaculture Research 47(11):3445-3454.

Allam B, Raftos D. (2015). Immune responses to infections. Journal of Invertebrate Pathology 131: 121-136.

In Preparation: Guo X, Wang G, Pales Espinosa E, del Castillo C, Tanguy A, Kraeuter J, Allam B. Identification of QPX-resistance markers by genome-wide candidate-gene association study in the hard clam.

- Presentations:
  - Oral

    Allam B, Pales Espinosa E, Wang G, Smolowitz R, Murphy D, Rivara G, Guo X. (2017). Development of strategies to mitigate QPX disease in the hard clam. Northeastern Aquaculture Conference and Expo. January 11-13, 2017. Providence, Rhode Island, USA.

    Guo X, Wang G, del Castillo C, Pales Espinosa E, Tanguy A, Kraeuter J, Allam B (2016). Identification of QPX-resistance markers by genome-wide candidate-gene association study in the hard clam. World Aquaculture Society Triennial Meeting, Las Vegas, NV. February 22-26, 2016.

    Allam B, Pales Espinosa E. (2016). Bivalve immunity and response to infections: Are we looking at the right place? International Society for Fish and Shellfish Immunology. Portland, Maine, USA. June 26-July 1, 2016.

    Dahl S, Allam B. (2015). Will Climate Change Help New York Hard Clams Fight Disease? Northeastern Aquaculture Conference and Exposition. January 14-16, 2015. Portland, Maine, USA.

    Allam B. (2015). Bridging basic and applied biological science in support of shellfish aquaculture. Long Island Shellfish Managers meeting. January 30, 2015.

    Dahl S, Allam B. (2014). Will climate change help New York hard clams fight disease? Meeting of the New York Marine Science Consortium. October 18, 2014.

    Dahl S, Barnes D, Allam B. (2014). QPX disease relationships with environmental parameters monitored over a decade in a Raritan Bay (NY) hard clam fishery. 106th Meeting of the National Shellfisheries Association. March 25-29, 2012. Jacksonville, Florida, USA.

    Allam B. (2013). QPX in hard clams: current disease status and mitigation strategies. New York State's Shellfisheries Advisory Committee. March 27, 2013. NY.

    Allam B. (2013). QPX disease in the hard clam: from fundamental research to disease management strategies. University of Rhode Island. November 2013.

  - Posters

    Wang K, Del Castillo C, Pales Espinosa E, Allam B. (2014). Clam focal and systemic immune responses to QPX revealed by RNA-Seq technology. 106th Meeting of the National Shellfisheries Association. March 25-29, 2012. Jacksonville, Florida, USA.

    Wang K, Pales Espinosa E, Allam B. (2014). Effect of “heat shock” treatments on QPX disease in the hard clam, Mercenaria mercenaria. 106th Meeting of the National Shellfisheries Association. March 25-29, 2012. Jacksonville, Florida, USA.

- Non-Peer-reviewed: Published abstracts:
  - Wang K, Del Castillo C, Pales Espinosa E, Allam B. (2014). Clam focal and systemic immune responses to QPX revealed by RNA-Seq technology. Journal of Shellfish Research 33(2): 661.
  - Wang K, Pales Espinosa E, Allam B. (2014). Effect of “heat shock” treatments on QPX disease in the hard clam, Mercenaria mercenaria. Journal of Shellfish Research 33(2): 661.

- Students dissertations:
  - Kailai Wang (2016). Molecular characterization of clam (Mercenaria mercenaria) immune responses against Quahog Parasite Unknown (QPX): Effect of host and environmental factors. http://gradworks.umi.com/10/13/10139857.html
  - Soren Dahl (2015). Ecology of QPX disease in the hard clam Mercenaria mercenaria. http://gradworks.umi.com/10/00/10000662.html

This website describes the project: http://you.stonybrook.edu/madl/research/mas_clam/

PROJECT COMPLETION REPORT
Genetic Marker-Assisted Selection of Northeastern Hard Clams for QPX-Resistance
Subaward # Z555103
Grant # 2012-38500-19656

Project Code: Subcontract/Account #

PART II

The hard clam or northern quahog, *Mercenaria mercenaria*, is one of the most valuable seafood products in the Northeast representing the first marine resource in several states. In addition to their economic value, hard clams, like other suspension feeding bivalves, play an important ecological role in benthic-pelagic coupling by transferring energy to the benthos and cycling large amounts of particulate matter. In recent years, the focus of the clam fishery has shifted from wild harvest to aquaculture production. Since the 1990's, several Northeastern states have suffered severe losses in aquacultured hard clam stocks due to a fatal disease caused by a protistan parasite called Quahog Parasite Unknown (QPX).

The overall aim of this project was to identify genetic markers associated with clam resistance to QPX disease. We screened a large *M. mercenaria* transcriptome dataset and identified genetic variants in key candidate immune genes. We further evaluated the association between these markers and resistance to QPX by evaluating variant frequency shifts after QPX mortality events. Results allowed the identification of 9 variants that hold promise for the development of marker-assisted breeding programs. In parallel, we evaluated disease resistance between different clam stocks and were able to show marked difference in resistance between Northeastern clam stocks.

Research Aim I. Identification of single nucleotide variants

Transcriptomic sequence data generated by our group from previous research (Wang *et al.*, 2016a) were compiled and screened for SNP detection. A total of 707 Million raw Illumina (100 bp in length) reads were filtered and trimmed according to length and quality score (min length 60 nt, end trimming quality 25, min quality filtering: 20 on 75 % of the read length) using the FASTX-Toolkit software v 0.0.13. rRNA cleaning was performed using the riboPicker software v 4.0.3 against SILVA database v111. High quality filtered sequence reads were subsequently used for de novo assembly using the de Bruijn graph assembler Trinity using the default parameters. Annotation of this de novo assembled transcriptome was performed using Blastx search against National Center for Biotechnology Information (NCBI) nonredundant sequences (nr) database with the E-value threshold setting at 1E-06. Putative gene functions were predicted by sequence similarity search against Gene Ontology (GO) database and assigning GO annotation terms to each mapped transcript. Protein domain search and enzyme annotation were also performed using InterPro scan and the Kyoto Encyclopedia of Genes and Genomes (KEGG). The annotated assembly was then exported to CLC Genomics Workbench bioinformatics software where variant searches were performed by mapping individual reads to the assembly using default parameters. A quality score of 30 was used to filter variants and only maintain high quality calls.

Assembled transcripts included a total of 66,378 contigs displaying single nucleotide variants, in addition to 568 and 426 contigs displaying deletion/insertion variants or multiple nucleotide variants, respectively (Figure 1). An additional 19,037 contigs simultaneously displayed 2 or more types of variations.

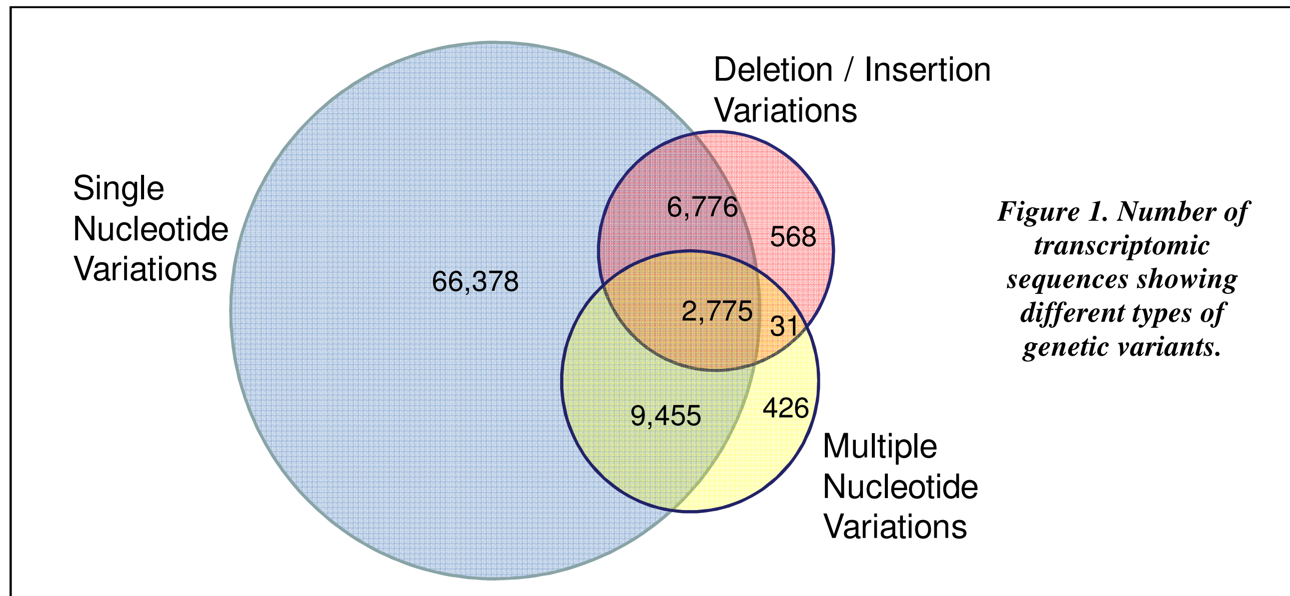

Figure 1. Number of transcriptomic sequences showing different types of genetic variants.

<u>Research Aim II. Identification of genetic variants associated with QPX disease resistance</u>

A total of 903 transcripts were identified to play a role in immune defenses and showed at least 1 type of genetic variation. These were further screened to identify those displaying single nucleotide polymorphism (SNP) variations that are appropriate for genotyping by sequencing. Therefore, 384 immune-related genes that have SNPs and conserved flanking sequences that are suitable for primer design were chosen for genotyping using the AmpliSeq method in conjunction with high throughput sequencing technologies. This technique allows the use of next generation sequencing technology for cost-effective genotyping of a large number of SNPs. Primer pairs were designed using the Ion AmpliSeq^{TM} Designer to amplify the candidate 384 genes. Primers were synthesized and pooled in a single-tube for multiplex amplification of each experimental sample. Among the 384 primer pairs, 373 pairs were shown to amplify target clam DNA. These were used for probing SNP frequency shifts associated with clam survivorship following QPX epizootics.

In this framework, the study contrasted SNP frequencies shifts in clams before and after exposure to QPX mortalities. Samples used in this work were generated from a previous field study described earlier by Kraeuter *et al.* (2011). Briefly, gill samples were obtained from three genetically-distinct clam stocks before deployment in Massachusetts and New Jersey. These included a clam strain from South Carolina, a strain from New Jersey, and a strain from Massachusetts. The deployed clams were sampled again after field mortalities (47 – 93%), caused

primarily by QPX. DNA was extracted from 56 - 64 clams from each group before pooled in equal amounts producing 3 before and 6 after-mortality DNA pools. The pooled DNAs were used as templates for amplification of the candidate genes with the AmpliSeqTM primer panel. Amplified products were purified before sequenced with the Ion Torrent PGM 400 bp module. Frequencies of each SNP were compared in samples collected before and after QPX-related mortality in a total of 6 paired comparisons, to identify significant or consistent changes.

Of the 373 genes targeted, 194 genes (52%) were successfully amplified and sequenced. Single-nucleotide polymorphisms (SNPs) and indels were identified and analyzed for post-mortality frequency shifts. About 0.55 Million reads were generated for each pool, averaging 850x coverage per gene. A total of 777 SNPs were identified in 140 genes, at a density of 1.4% or 1 SNP/70 bp. Among these, a total of 70 SNPs showed significant allele frequency shifts before and after field deployment, with 9 SNPs in 7 genes showing consistently allele-frequency shifts across all clam strains in both field sites (Table 1). The genes include interferon-induced guanylate-binding protein 2 (GBP2), cytochrome b-245 light chain-like (CYB245), a metalloproteinase domain-containing protein 10 (MP10), inhibitor of apoptosis 1 (IAP1), sparc-related modular calcium-binding protein 1-like (SMOC1), hemagglutinin/amebocyte aggregation factor (HAAF), and programmed cell death protein 7-like (PCDP7).

Table 1. SNPs with allele-shifts in the same direction (9 SNPs in 7 genes). Stock-State combinations are shown in separate columns (e.g. M-MA and M-NJ designate the Massachusetts clam strain deployed in Massachusetts or New Jersey, respectively).

				After/before frequency ratio, stock-state						
Gene	**Position**	**Ref**	**Variant**	**M-MA**	**M-NJ**	**N-MA**	**N-NJ**	**S-MA**	**S-NJ**	**P-value**
GBP2	28	C	T	0.67	0.84	0.88	0.86	0.85	0.74	0.0029
CYB245	117	G	A	0.80	0.78	0.90	0.67	0.88	0.84	0.0023
MP10	83	C	T	0.68	0.78	0.83	0.68	0.82	0.74	0.0004
	127	G	A	0.72	0.90	0.74	0.65	0.85	0.92	0.0073
	244	A	G	0.77	0.84	0.75	0.76	0.79	0.85	0.0002
IAP1	116	G	A	0.77	0.85	0.94	0.65	0.63	0.97	0.0074
SMOC1	171	-	A	0.96	0.88	0.96	0.91	0.94	0.77	0.0094
HAAF	72	C	T	1.07	1.12	1.03	1.01	1.03	1.08	0.0084
PCDP7	486	A	T	1.48	1.99	1.25	1.04	1.29	1.27	0.0088

In particular, a novel mutation in a gene involved in the homeostasis of reactive oxygen species (CYB245) was shown to be associated with increased clam survival (Figure 2). This mutation represents a prime candidate for the validation of genetic markers associated with QPX resistance.

Figure 2. Enhanced clam survival was associated with a novel non-synonymous mutation in CYB245 gene.

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Mitochondrial targeting sequences   *******8***
Saccoglossus kowalevskii            MGQIEWAMWANEQALASGAIICVGGIIGVNG-FTGWEFGVYAIIAGFLICILEYPRSRRV
Branchiostoma floridae              MGQIEWAMWANEQAIISAWVMLTGGIIGLTG-FNRWEIAAYSVAAGIFIILLEYPRGKRK
Takifugu rubripes                   MGKIEWAMWANEQALASGFILLTGGVVGVAGQFRGWQFAAYAVAAGVLVCLLEYPRSKRS
Danio rerio                         MAKIEWAMWANEQALAAGLIYLTGGIVGVAGQFRGWQFAAFGIAAGVFVCLLEYPRSKRG
Xenopus (Silurana) tropicalis       MGQIEWAMWANEQALASGLILLTGGIVAVAGQFKGWQFGAYGVAAGVFITLLEYPRSKRK
Xenopus laevis                      MGQIEWAMWANEQALASGLILLAGGIIAVAGQFKGWEFGAYGIAAGAFITLLEYPRSKRK
Rattus norvegicus                   MGQIEWAMWANEQALASGLILITGGIVATAGRFTQWYFGAYSIVAGVLICLLEYPRGKRK
Homo sapiens                        MGQIEWAMWANEQALASGLILITGGIVATAGRFTQWYFGAYSIVAGVFVCLLEYPRGKRK
Aplysia californica                 MGKIEWAMWANEQAIASSCVTALGGFIAAIGQFKNWQIGVYAIAAGVLTFALEYPRGKRQ
Priapulus caudatus                  MRQIEWSMWANEQALTSALLTFIGGVMGITQVFKNWGFGLYGIIISILVGLFEYPRGKRM
Crassostrea gigas                   MRQIEWSMWANEQAIISSSVVLFGGIIGITGFFRAWEIGIYAVVVAVLVFVIEYPRGKRA
Mercenaria mercenaria ref           MSQIEWAVWANEQALTSCSVLLLGSAVGIAGMFNRWQFGIYGLIASLFILVIEWPRSKRK
Mercenaria mercenaria alle          MSQIEWAMWANEQALTSCSVLLLGSAVGIAGMFNRWQFGIYGLIASLFILVIEWPRSKRK
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Most SNPs that shifted in opposite directions may represent sampling or genotyping artifacts. However, some SNPs may be linked to the resistant allele in opposite phases in different populations and therefore shift in opposite directions. Some of the genes that display opposite shifts in different clam strains and/or field sites are given in Table 2. They include tumor necrosis factor ligand superfamily member 6 (TNF), TNF receptor-associated factor 7 (TRAF7), and transforming growth factor-beta receptor-associated protein 1 (TGFBR1). If these opposite shifts are indeed caused by opposite linkage phase, they can still be used for marker-assisted selection. Further confirmation is needed.

Table 2. SNPs with allele-shifts in different directions. See legend of Table 1 for details.

				After/before frequency ratio, stock-state					
Gene	**Position**	**Ref**	**Variant**	**M-MA**	**M-NJ**	**N-MA**	**N-NJ**	**S-MA**	**S-NJ**
TNF	126	T	C	**1.62**	**1.26**	**1.29**	**1.11**	**0.54**	**0.74**
	170	C	T	1.48	1.17	1.26	1.10	0.61	0.76
	226	T	C	1.28	1.10	1.13	1.02	0.71	0.78
TRAF7	306	A	T	1.02	1.24	0.79	0.79	1.40	1.35
	337	A	G	1.01	1.23	0.79	0.78	1.38	1.28
	423	T	C	1.30	1.60	0.60	0.61	2.21	1.96
	430	C	T	**1.32**	**1.61**	**0.61**	**0.61**	**2.29**	**2.02**
TGFBR1	109	A	T	0.77	0.97	0.81	0.98	1.01	1.22
	226	C	T	0.74	0.92	0.80	0.99	1.06	1.24
	256	T	C	0.64	0.90	0.77	0.96	1.07	1.27
	314	AA	-	**0.55**	**0.89**	**0.74**	**0.94**	**1.16**	**1.33**

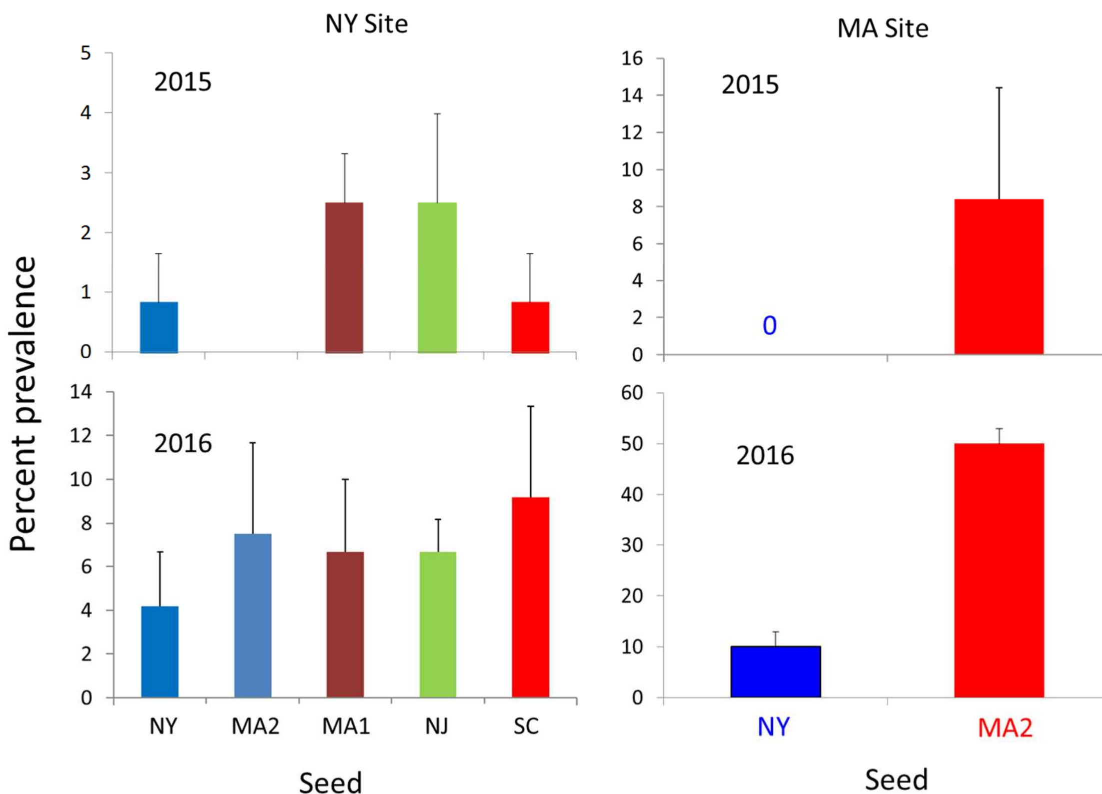
Research Aim III. Identification of resistant Northeastern clam stocks

Three strains of QPX survivor clams resulting from a prior NRAC-funded project (to Dr John Kraeuter *et al.*) were transported to the Suffolk County Marine Environmental Learning Center (SCMELC) in Southold, NY where they were conditioned for spawning as per industry standard. Twenty clams from each group were individually spawned, with different strains kept in separate tanks. Larvae were cultured using industry-standard techniques and post sets were grown in land-based upwellers before being placed in a floating upweller system. We also secured seed from 3

commercial sources (1 from each of the following states: MA, NY and NJ) to be included in the study. All seed was tested for pathology before transfer to field sites and 1 of the commercial seed (NJ strain) was tested positive for QPX and hence could not be imported for field deployment in NY and MA. Therefore, a total of 5 clam strains were deployed in a field site in NY in early July 2014 including the 3 custom-spawned clams (SC, NJ and MA) and 2 commercial strains (NY and a second MA strain hereby designated MA2). Clam deployment in the MA site was limited, however, to the 2 commercial strains (NY and MA2) since there were no sufficient custom-spawned clams left (because of higher than expected mortality during the harsh winter of 2014) to ensure a statistically-robust field deployment in MA (3 replicate plots each). Deployed clams were sampled in June (NY) and October (MA) 2015 and October 2016 (both sites) for the assessment of QPX disease prevalence.

Results showed the presence of QPX in the deployed clams in both field sites but higher prevalence was noted in the MA site (Figure 3). Significant differences between strains were noted in the MA site only with disease prevalence being markedly higher in the MA2 strain as compared to the NY strain. In parallel to disease prevalence, mortality rates were also higher among the MA2 clams deployed in MA as compared to their NY counterparts (Figure 4). These findings are counter

Figure 3. QPX disease prevalence in different clam stocks deployed in NY (left panels) and MA (right panels) and surveyed in 2015 (top row) and 2016 (bottom row). *Significantly higher disease prevalence was measured in the MA2 clam strain deployed in MA as compared to the NY strain deployed in the same site (note that different panels have different Y-scales).*



intuitive as the expectation was that local strains would perform best in local environments, highlighting the need for a better understanding of genetic x environment interactions in aquaculture operations.

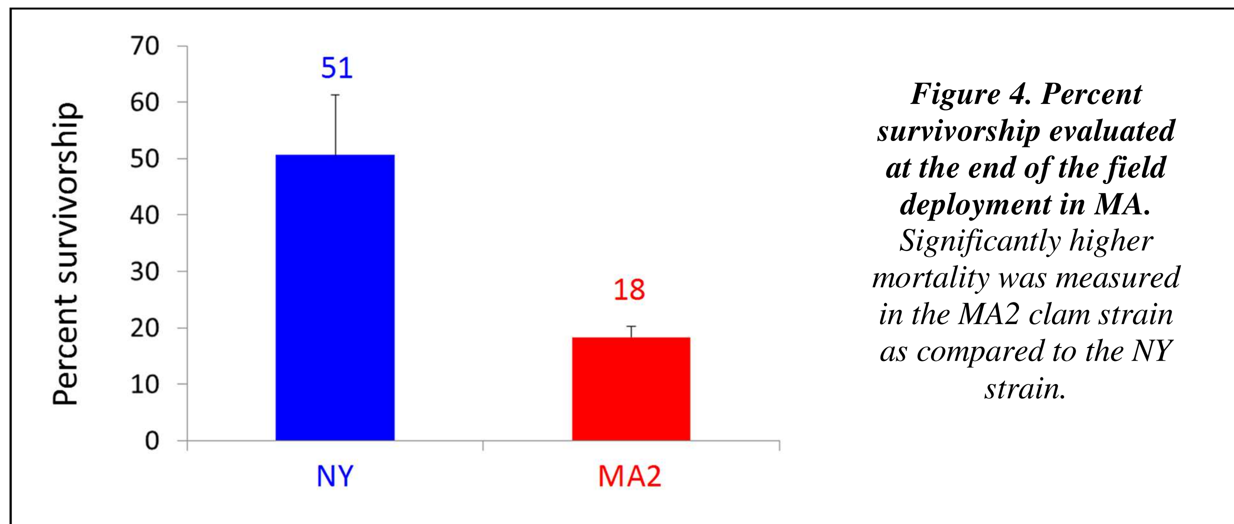


Figure 4. Percent survivorship evaluated at the end of the field deployment in MA. *Significantly higher mortality was measured in the MA2 clam strain as compared to the NY strain.*

Genetic material from survivors and pre-deployment stocks are currently being used in the framework of a new project funded by the USDA to validate genetic markers identified under Research Aim II.

Discussion/Comments

A genetic basis for clam resistance to QPX disease has been previously reported during field (Ford *et al.*, 2002; Ragone-Calvo *et al.*, 2007; Dahl *et al.*, 2010) and laboratory (Dahl *et al.* 2008) trials. The finding of genetic bases for QPX resistance is not surprising as host-defense against pathogens is controlled by many genes, and variation at these genes leads to differences in resistance and survival. Results from the present study support this scenario and allowed, for the first time, the identification of nine genetic variants potentially associated with QPX resistance.

Some of the identified genes carrying significant mutations play a central role in immunity. For example, guanylate-binding protein 2 (GBP2) is an essential part of the interferon-induced defense in vertebrates and is a primary player in antiviral immunity. Its role in immunity has been recently expanded as it was shown to include confer resistance against infection by bacterial pathogens (*Listeria monocytogenes* and *Mycobacterium bovis*; Kim *et al.*, 2011) as well as the protozoan parasite Toxoplasma gondii (Degrandi *et al.*, 2013). Similarly, different members of the metalloprotease family have been shown to play important roles in fundamental physiological processes, such as cell proliferation, differentiation, adhesion, migration, apoptosis, and inflammation (Le *et al.*, 2007; Vanlaere and Libert, 2009). In the hard clam, Wang *et al.* (2016a and b) showed a significant upregulation of several metalloproteases in response to QPX infection and suggested these to play a primary role in clam immunity and resistance to the infection. In this

context, mutation in MP10 gene may regulate clam resistance to the infection. Finally, cytochrome b-245 is a primary component of the microbicidal NADPH oxidase system of phagocytes and mutation in this gene can lead to alteration in the ability of blood cells to produce cytotoxic reactive oxygen species, leading to increased susceptibility to microbial infections (Panday *et al.*, 2015; Bast *et al.*, 2017).

Interestingly, our results showed that some markers displayed divergent selection at our two experimental sites, indicating allele-specific local adaptation. These findings are not surprising in light of a growing body of evidence supporting genotype-environment interactions in aquacultured stocks. This complexity has been highlighted by recent studies in oysters by Frank-Lawale *et al.* (2014) who evaluated a large program for the development of oyster lines in Virginia. Similar findings were also reported by Proestou *et al.* (2016) who deployed selected oyster lines from five geographic areas (ME, RI, CT, NJ, and VA) across the Northeast and mid-Atlantic coasts. In this context, additional studies are needed to understand the basis for the divergent selective pressure on the various loci.

Overall, the genetic variants identified in this study hold promise for marker-assisted selection (MAS) of QPX-resistant clam stocks. MAS provides several advantages as compared to traditional selective breeding of survivor clams. For example, exposure to diseases in the field is highly variable, and some clams may survive by chance rather because they are genetically resistant. Similarly, disease pressure may be absent in some years when breeding decisions have to be made. Finally, typical commercial hatchery practices have been shown to result in a small number of individuals contributing to the gene pools of cultured populations, reducing genetic variability and leading to inbreeding depression. With genetic markers for resistance, selection could continue when disease pressure is low. For these reasons, MAS has been a popular choice for producing resistant varieties of aquacultured species such as the Japanese flounder (Fuji *et al.*, 2007; Ozaki *et al.*, 2012) and the Atlantic salmon (Moen *et al.*, 2009) and is a very appealing approach for shellfish selection.

In this study, we report a better resistance to QPX disease and resulting mortalities in the MA field site among a NY clam strain as compared to a strain that originated from MA (MA2). These findings are intriguing since MA has been heavily hit by QPX epizootics since the 1990's while QPX disease has not been a major hamper to clam aquaculture operations in NY (wild clams are more severely hit by QPX than aquacultured clams in NY despite episodic development of the disease in aquacultured stocks; Allam, unpublished). These findings highlight the need for a better understanding of genotype x environment interactions among aquacultured stocks and warrant a more thorough evaluation of the benefits of integrating the NY line in breeding programs throughout the Northeast.

In summary, our study allowed the identification of genetic variants associated with clam survivorship following QPX epizootics. These variants need to be validated as markers for resistance before being proposed for marker-assisted selection. Such outcome will represent a major progress in mitigating the devastating effects of QPX disease on hard clam aquaculture in the Northeast.

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FINAL REPORT

Project Title	Impact assessment of NRAC funding on aquaculture in the Northeast
Reporting Period	9/1/15-6/30/17
Author (Chair)	Name of person submitting this report. Elizabeth Fairchild
Key Word	northeast, aquaculture, evaluation, research, economic, impact
Funding Level	Total funds allocated for this project to date. *Year One: FY 2015, $67,614*
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Project Objectives	List each objective **Objective 1:** Review the effectiveness of NRAC-funded projects (using targeted interviews and in-depth data collection and analysis) to assess their impacts to aquaculture permitting, siting, production, disease management, and harvesting programs across cultured species and production systems (inland, coastal, closed, integrated, etc.).

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	Objective 2: Summarize and describe lessons learned and outcomes (including, where possible an estimate of the return on investment) from projects funded through the NRAC RFR and Workgroup processes. **Objective 3:** Evaluate the effectiveness of project approaches to promote solutions for aquaculture source problems. **Objective 4:** Produce science-based knowledge that can be utilized to set new funding priorities, which will yield more effective aquaculture research, education, and extension programs funded by NRAC. **Objective 5:** Provide examples where the NRAC funding mechanisms have worked synergistically or where they have failed to develop synergies. **Objective 6:** Deliver results of the synthesis to NRAC, the science community, and relevant stakeholder groups (e.g., industry, municipal, state and federal agencies, commodity organizations).
Anticipated Benefits	State briefly how the project will benefit the aquaculture industry – directly or indirectly. The overall objective of this work group is to assess the scientific, socio-economic, and policy impacts of accomplishments made through NRAC's portfolio of recently (2005-2014) funded 32 aquaculture projects, including extension workgroup projects. Incorporated in this synthesis will be how these projects have or have not helped move the aquaculture industry close to solutions for the diversity of problems it faces within the region. From the resultant information, suggestions for achieving higher impacts will be identified that NRAC should consider in future funding initiatives to benefit the Northeast aquaculture industry better.
Project Progress	Summarize concisely for each objective the progress toward accomplishment to date. This has an 8,000 character limit. Objectives 1-3: The UNH Survey Center and the Carsey School of Public Policy's Evaluation Program evaluated the relevancy, usefulness, and impact of NRAC-funded research and work group projects to aquaculture industry stakeholders by querying diverse groups and analyzing their responses. Amongst questions asked were: 1) What are the critical scientific and management findings of the NRAC portfolio (since fiscal year 2005)? 2) What is the overall impact to the industry and local and regional economies with respect to return on investment?

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	3) How effective have integrated (research, education, and extension) projects been at moving stakeholders (e.g., industry) closer to solutions for diversified issues in inland and coastal systems, including diversification of the industry? 4) What synergies developed among projects that resulted in improved leveraging of resources and accelerated movement toward solutions? 5) What issues should NRAC address as science-industry priorities in the future and what is the best means to address those issues through research, education, and extension efforts? Working with the PC and Aquaculture Advisors, a focus group was held to formulate survey contents at the beginning of Year 1 to ensure that the evaluation addressed all the relevant stakeholders, themes, and aspects of the NRAC program throughout the evaluation. A series of meetings were held with the UNH research team to develop questionnaires that provided data needed to address research questions, including estimates for return on investment of NRAC research programs. From these meetings, the sampling frame for each population of interest (NRAC grantees, their industry collaborators, and industry stakeholders) was finalized. Draft questionnaires were developed based on input from the Project Team, including the Aquaculture Advisors, and the surveys were tested by the Aquaculture Advisors to ensure clarity of questions, ease of understanding, ease of completion, and comprehensiveness of the questionnaire for achieving research goals. Whenever possible, identical questions were used across survey populations to understand how different groups view the utility of NRAC grants. Three surveys were designed, conducted, and completed. The first survey targeted Project Coordinators (n=32; response rate=100%) for each of the NRAC-funded aquaculture projects under review. The second survey targeted named collaborators and stakeholders identified in individual project proposals and documents or specifically named by PIs (completed by 141 people; response rate=52%). This group included both funded and non-funded participants which included extension, industry, and other researchers. A third survey targeted a broader array of aquaculture industry stakeholders to provide a more unbiased (by NRAC experience) view of the research needs and priorities of the aquaculture industry as well as the practical utility and scalability of NRAC research projects. This group was constructed using state supplied lists of licensed, private aquaculture growers or propagators (completed by 273 businesses; response rate=28%).

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	Prior to receiving the survey, respondents were notified via emails sent by both the PC and the NRAC Director, on behalf of the Project Team, to expect a second email from the Project Team with a link to the survey and encourage them to complete the survey when it arrived. Each respondent received an initial email describing the project, an email directing them to a web site to complete the questionnaire, and up to three follow-up emails as needed. Those who were unable or did not respond to web surveys were contacted by telephone to remind them of the project and to direct them to the web site to complete the survey or the survey was completed over the phone. All three surveys were analyzed across data sources for similar themes, differences, categories, and content, by all participating stakeholders and by different groups of stakeholders. Data from across all these sources were analyzed to assess the relevance, usefulness, and impact of NRAC-funded research to the aquaculture industry. In addition, a content analysis of original project proposals, progress reports, and, when available, final and impact reports written and submitted by project PCs to NRAC were reviewed for all 32 research projects to determine expected and realized project impacts and tabulate output metrics. For five projects still active at the time of this assessment, project impacts were not able to be determined, however some output metrics could be quantified based on submitted progress reports. An input-output model of each state in the NRAC region economy was created to estimate the economic impacts that resulted from the completed NRAC projects within each state where an NRAC project occurred and at the regional level. Objectives 4-5: During this reporting period, all data were analyzed to produce science-based knowledge about the effectiveness of the 32 NRAC-funded projects, determine what economic impacts they've had on the regional economy, and illustrate examples where the NRAC funding mechanisms have worked synergistically and where they could be improved. A report of all study findings was initiated and will be completed with the continuing award for this project. Objective 6 has not been started yet but will be accomplished in the following year with the continuing award for this project.
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. The results have not been extended to the intended users yet.

Targeted Audiences	Provide information on the **target audience** for efforts designed to **cause a change in knowledge, actions, or conditions**. • Policy makers will have a better idea of how past NRAC research projects have affected the economic livelihood of people involved in the aquaculture industry and the resulting ripple effects through the economy. With economic impact results, policy makers can direct policy and key areas for future research better. • The NRAC leadership will know which research priority areas will be of key funding importance to increase aquaculture growth in the northeast. • Researchers will be informed of these key issues which most limit aquaculture growth so that they can work towards solutions with the industry. • The Northeast aquaculture industry will be made more aware of the role NRAC has had in applied aquaculture research. • Other RACs will be able to use this assessment template to conduct their own impact studies.
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. • In Year 1, a meeting was held at UNH that included the UNH team and the Aquaculture Advisors to develop and review survey methods and design. • Three types of on-line surveys were designed to target researchers, their collaborators, and the Northeast aquaculture industry to determine the effectiveness of NRAC-funded projects. • In Year 2, a meeting was held in conjunction with the Aquaculture Advisors in conjunction with NACE to review progress on the project to date.
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.** Nothing to report yet.
Impacts Summary	Provide short statements (2-3 sentences) about each of the following:

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<table>
<tr><td></td><td>(pre-established fields for Researchers to complete short statement answers)
1. Relevance: Issue – what was the problem?
NRAC has invested over $5 million in aquaculture research since 2005, however its effect is unknown. To determine the outcome of NRAC funding on the northeast aquaculture sector, there is a need for an impact assessment.
2. Response: What was done?
Research is in progress to determine the impact $4.1 million invested by NRAC during 2005-2014 has had on the Northeast aquaculture industry. A complete synthesis will be provided with the continuing award for this project.
3. Results: How did your work make a difference (change in knowledge, actions, or conditions) to the target audiences?
Nothing to report yet.
4. Recap: One- sentence summary
Nothing to report yet.</td></tr>
<tr><td>Publications</td><td>Nothing yet.</td></tr>
<tr><td>Students/Participants:</td><td>No students have worked on this project.</td></tr>
<tr><td>Partnerships</td><td>List any partners that you worked with on your project. Provide the following information for each Partner:<table><tr><td>Partner</td><td>Specific Type
Type</td><td>Level
Level</td><td>Nature of Partnership</td></tr></table></td></tr>
</table>

NRAC FINAL REPORT

Project Title: White worm, *Enchytraeus albidus*, production and marketing for live aquaculture feed

Reporting Period: 9/01/2015- 8/31/2017

Author (Chair): Elizabeth Fairchild

Key Words: white worm, aquaculture, live feed, marine, freshwater, finfish

Funding Level Total funds allocated for this project to date.

Year One: FY 2014, $76,646
Year Two: FY 2015, $75,279

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PROJECT OBJECTIVES

Objective 1: Measure the effects of different feeds and production lengths on white worm growth, reproductive potential, and nutritional composition.

Objective 2: Evaluate the effects of rearing container size and/or shape for white worm production.

Objective 3: Characterize and evaluate white worms as a live feed for multiple aquatic species.

Objective 4: Evaluate the nutritional composition of white worms fed different enrichment products.

Objective 5: Improve white worm production potential.

Anticipated Benefits (how the project will benefit the aquaculture industry – directly or indirectly)

This research project (1) developed "modern" white worm production protocols, which eventually could be adapted for commercial scale production, and (2) worked with many interested aquaculture sectors to identify the white worm market(s) through a series of workshops, surveys, and testing by aquaculturists, resulting in identifying the target markets and providing worms nutritionally customized for those consumers (species). This research promotes sustainable, environmentally friendly tactics in its use of recycled, local, waste by-products for worm feed, and a low carbon footprint. This research may yield economically viable techniques for those aquaculturists looking to diversify and a readily-available product for the aquaculture market.

PROGRESS ACHIEVED COMPLETING OBJECTIVES

OBJECTIVE 1: Measure the effects of different feeds and production lengths on white worm growth, reproductive potential, and nutritional composition.

White worms are notoriously unfussy when it comes to feeding protocols; worms will survive on just about anything including cooked vegetables, baby cereal, stale fish feed, hot dog buns, and coffee grinds. This diet flexibility is one of the main advantages of white worm production; however, we do not know which feed promotes the fastest growth and production. Evaluating and determining which feeding protocols (optimal feeds and culture period before harvest) is paramount to developing large-scale and cost-effective white worm production techniques.

Our aim is to produce a live feed that can easily and cheaply be incorporated into the routine of aquaculturists, thus, the following dietary experiment was framed to distinguish the most optimal, locally-sourced feeds available to most coastal communities. We examined white worm potential as “local recyclers” by conducting a common garden experiment testing five feed treatments (coffee grinds, brewery wastes, stales [old bakery products], produce, and sugar kelp grown at UNH) over the course of different production cycles (6, 9, 12 wks) during Year 1 of the project. At the end of each production cycle, the worm population and reproductive output were calculated from each replicate. In addition, to evaluate the effects of feed and production cycle length on worm nutrition, subsamples of the worms from each experimental unit (n=45) were shipped to and analyzed by Dr. Jesse Trushenski (Southern Illinois University, Carbondale) for proximate composition and fatty acid profiles, at the beginning and at the end of the experiment.

We found that feed type and production cycle duration affected white worm biomass, reproductive potential, and proximate and fatty acid composition. In general, white worm cultures fed coffee grounds, stale bread, and spent brewing grains had higher production yields than cultures fed mixed produce or sugar kelp. Dependent on feeds and production cycle duration, white worms were high in protein (49-69%) and lipids (10-27%) and low in ash (5-8%), indicating that they would meet the dietary needs of species requiring a high protein, relatively high lipid, low ash diet. Compared to fatty acid profiles reported for standard live feeds like rotifers, *Artemia*, and copepods, white worms provided less n-3 long-chain polyunsaturated fatty acid content (DHA 0-0.5%, EPA 2-18%, total LC-PUFAs 4-25%), with the highest levels in worms fed mixed produce or sugar kelp. White worms exhibit many attractive characteristics as feeds, but commercialization will require improved culture techniques to produce greater worm biomass while reducing production costs. Depending on the target species, white worms may need enrichment to increase n-3 LC-PUFA levels.

This study was published in *Aquaculture* and the paper is included with the final report.

OBJECTIVE 2: Evaluate the effects of rearing container size and/or shape for white worm production.

This objective, examining the effect of culture container shape and size on worm productivity, was not completed. During the course of the project, we realized that this experiment was much less critical to evaluating the potential for using white worms by the aquaculture industry. Instead, we focused our resources on protocols that need to be determined in order to successfully complete Objective 3 – mainly establishing shipping and receiving guidelines for our worm testers. In addition, we became acutely aware while harvesting live worms for analyses, that coming up with more efficient harvesting techniques is the key bottleneck to scalability of white worms. We diverted funding from Objective 2 to partially support a graduate student to work exclusively on Objective 5 – improving white worm production potential.

OBJECTIVE 3: Characterize and evaluate white worms as a live feed for multiple aquatic species.

White worms were characterized and evaluated as a live feed for multiple aquatic species through a series of steps.

Biosecurity concerns:

White worm diagnostic testing with Dr. Giray at Kennebec River Biosciences was completed to formulate and provide pathogen screening strategies for white worms to ensure we provide a bio-secure product for the aquaculture industry. All viral, bacterial, and parasitic assays were negative, and results were shared with worm testers and presented in a poster at Aquaculture America 2016.

Live white worm shipping and receiving protocols:

Prior to shipping live white worm samples to industry stakeholders for testing and feedback, we wanted to ensure the worms would arrive in good condition. A series of three 'test' shipments of live worms were sent from UNH to co-PI Dr. Michelle Walsh at the Florida Keys Community College (Key West, FL) in Jan. 2016. Test 1 was shipped via 2-day priority mail, took 3 days to reach its destination, and when it arrived, the water temperature was 18 °C and most worms were dead. Tests 2 and 3 were shipped simultaneously by overnight FedEx in bags injected with oxygen. The only difference between the test shipments was one sample was shipped in a semi-permeable bag and the other in a standard polyethylene fish bag. Both test samples arrived the following morning, temperature was 3 °C, but survival was higher in the polyethylene bag. From this trial and error, we determined that live white worms needed to be overnight shipped to ensure high survival upon receipt and the polyethylene bags worked better than the semi-permeable bags we tested. We then wrote up receiving guidelines for our industry stakeholders based on UNH institutional knowledge of successful white worm handling practices plus the following experiment to determine the shelf-life of white worms kept in freshwater.

Methods:

A factorial experiment was conducted twice to evaluate the effects of time (0 -14 days) and water treatment (daily water changes or no water changes) on harvested worm survival in freshwater. Each combination (water treatment x day) was replicated in triplicate (2 x 15 x 3 = 90 experimental units). Approximately 3 grams of worms were harvested from one worm culture for stocking out the experiment. Experimental units consisted of 90 25-ml beakers filled with 20 ml distilled room temperature water. Twenty (20) live white worms were added to each beaker and all beakers were placed in a refrigerator set to the middle of the temperature range. To assess the temperature experienced by the worms, one datalogger that recorded temperature hourly (Onset Computer Corporation, Bourne, MA) was placed in a beaker in the refrigerator filled with distilled water during Run 2.

Each day for up to 14 days, a total of six beakers, three from each water treatment, were removed from the fridge, allowed to "warm up" to room temperature so that the live worms would start moving, and the number of live worms were counted (Fig. 3.1). These beakers then were removed permanently from the experiment. The remaining beakers that received daily water changes were removed from the refrigerator and a dropper was used to remove the

existing water and replace it with 20 mls of refrigerated, new, distilled water. After water changes, these beakers then were returned to the refrigerator until the following day. White worm survival for each Run was analyzed by two-way ANOVA testing for the effects between water treatment and time post harvested (JMP Pro 12.2.0). All effects/differences were considered significant at $P < 0.05$.

Results:

Run 1 began on 7/26/16 and ran for 14 days; Run 2 began on 9/20/16 and ran for 10 days. Overall mean water temperature during Run 2 was 3.8 ± 0.5 °C, and remained between 3-5 °C every day.

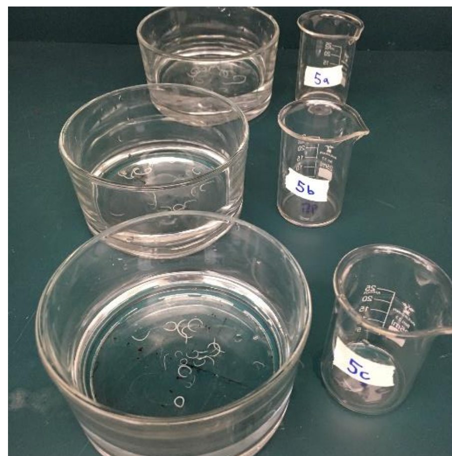


Figure 3.1. *White worms from one water treatment warming up to room temperature for daily survival assessment.*

White worm survival in both Run 1 and Run 2 was impacted by the effect of time on the water treatment (water change, no water change; $p<0.001$). Although mean worm survival began declining the day after harvest, there were no significant differences between worms that experienced water changes and those that did not until day 3 in Run 1. After day 3, survival plummeted in worms that did not receive water changes (16% survival – no water change, 93% survival – water change; $p<0.001$; Fig. 3.2). However changing water did not necessarily prolong the shelf-life of the worms. By day 4, worms from both water treatments had equally poor survival and increased mortality for worms that had been receiving water changes continued until no worms remained alive by day 6.

In Run 2, the same initial mortality pattern was observed but with worm survival decreasing quicker in the first few days; by day 1 survival had decreased to 65% and by day 4, survival was 0% in the water change treatment. For worms that did not experience water changes, survival was higher but very variable between replicates and over time (Fig. 3.2). A gradual decline in survival occurred immediately resulting in >10% mortality each day over the first 3 days. Afterwards, variability was high, usually due to one replicate dying off in entirety.

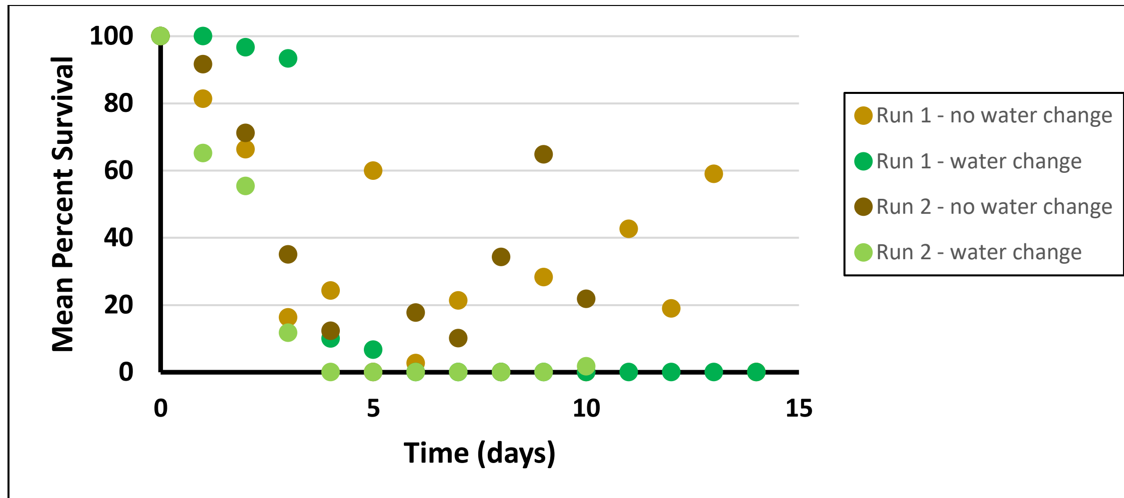


Figure 3.2. *Mean percent survival of white worms stored in refrigerated distilled water over time (error bars are not depicted). Brown circles denote vessels that did not have water changed over the course of the study (run). Green circles denote vessels in which all water was replaced daily.*

Implications:

The methods of this experiment differed somewhat from how we packaged white worms for shipment to the industry stakeholders. To get industry stakeholder feedback, approximately 7,000 worms (10 g) were harvested and gradually chilled in the refrigerator over several hours in an open pitcher of 1L distilled freshwater. Just prior to shipping, the worms and water plus another 1L chilled water were gently poured into a polyurethane fish bag, infused with oxygen, and secured in an insulated shipping box with several frozen cold packs. Samples were shipped overnight by FedEx with instructions for use and the pathogen screening results enclosed. While we tried to mimic the proportion of worm quantity to water volume of samples sent to industry stakeholders, the small 20 ml beakers used in this experiment may have created an adverse effect on worm survival in that the worms experienced colder conditions for longer periods of time relative to the worms in the larger (2L) samples. This should be evaluated to determine a more precise shelf life of the white worm samples, as well as the effects of water temperature on worm survival over time, and if longer term survival would improve with less frequent water changes. However, to err on the side of caution, we used these results to formulate our recommendations to our industry collaborators: upon receiving the white worm samples, the worms should be stored in a cool place, like a refrigerator, until use. Up to a 50% water change could be done initially if the shipment had gotten warm (>10 °C) during transport or water quality seemed poor, but otherwise was not necessary. Shelf life could not be guaranteed beyond three days so we advised our collaborators to use the white worms within 1-2 days of receiving the shipment.

Industry feedback and white worm potential:

Based on stakeholder input from the workshop held in Year 1, a survey was created for evaluating white worms as a live feed. Most original non-funded participants plus several new aquaculture stakeholders, who we connected with via word of mouth and as an outcome from giving presentations, tested live white worms in their facilities. Ten-gram samples (~7000 worms) were overnight-shipped to anyone in the US requesting worms. A total of 21 samples were shipped to 18 participants resulting in approximately 222,530 worms given out for industry feedback. Forty-one percent (41%) of participants fully completed an online survey detailing their experiences using the white worms as well as information on their facilities (e.g., species cultured, volume, live feed needs, etc.), and 47% partially completed the survey. The surveys were analyzed to determine which species or sector(s) of the aquaculture industry are most likely to benefit from using live white worms.

Survey results:

A total of 18 white worm testers either partially or fully completed the online survey. The majority of testers did not represent commercial entities but worked in an academic or research facility or in a government facility (Fig. 3.3). Several commercial facilities that we reached out to were supportive of the white worm research and interested in the results, but did not want to expose their facilities to any possible contamination from an 'unknown' product. The commercial entities that did agree to test white worms grew ornamental species only.

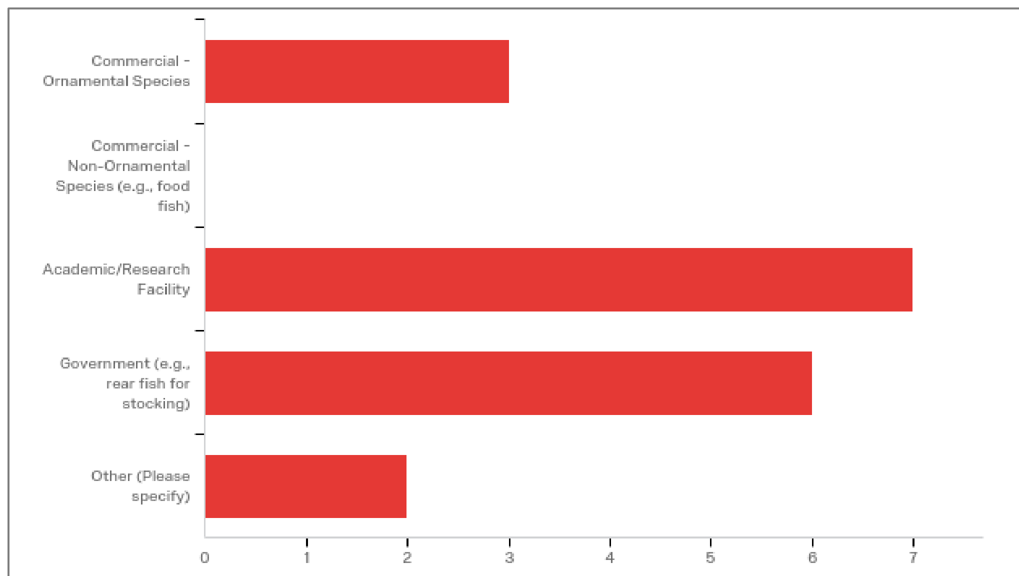


Figure 3.3. *Aquaculture sector classification of the 18 white worm testers who took the industry feedback survey. The other category includes one person in "government environmental research" and one person in international extension.*

The majority (72%) of facilities raised marine species though many also raised freshwater (39%) and brackish/euryhaline species (28%; Fig. 3.4). Almost all species (94%) were cultured in water ≥ 15 °C, with only 17% classified as cold-water species (< 15°C). These aquaculture facilities employed mostly recirculating (72%) and flow-through (50%) systems, with a few facilities (17%) using outdoor ponds too (Fig. 3.5).

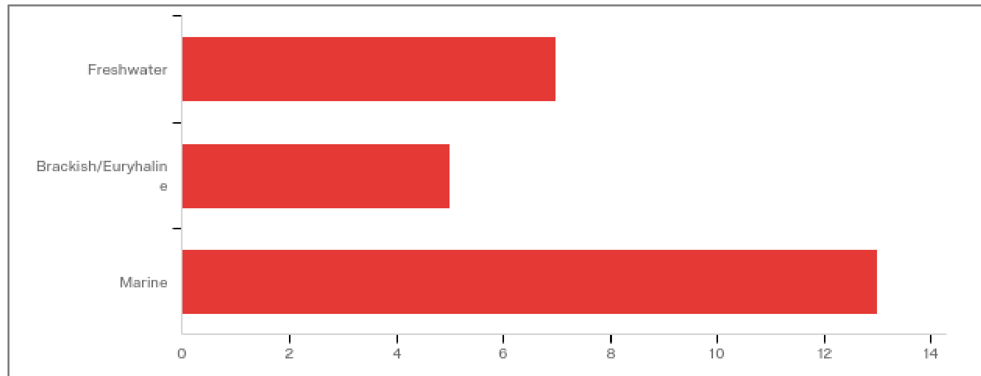


Figure 3.4. *Primary water type of facilities where white worms were tested (multiple answers possible).*

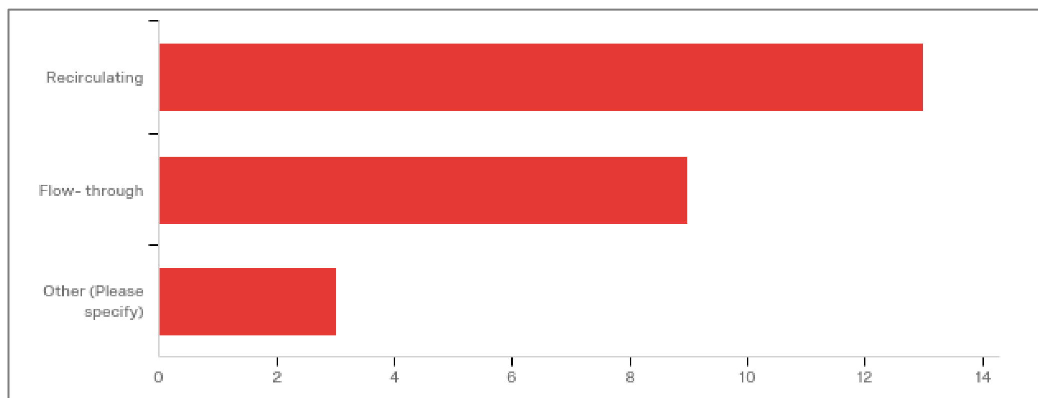


Figure 3.5. *Type of systems used in the facilities where white worms were tested (multiple answers possible).*

Of the facilities that used live feeds, 71% grew their own live feed while the remainder purchased the feed. All used rotifers and *Artemia*, 42% used copepods, and 33% used worms (blackworms, microworms, or polychaetes). Worm users grew microworms and polychaete worms in house but purchased blackworms from a supplier. White worm samples also were sent to facilities that typically don't use live feed in their normal operations; one-third (33%) of testers fell into this category.

White worms were offered to 31 marine and freshwater fish species of various life history stages, plus three bird species in a shoreline exhibit in a public aquarium (Table 3.1). The majority of fishes (58%) were ornamental fishes.

Table 3.1. *Organisms white worms were fed to and their responsiveness to the white worms when offered the first time and after multiple times, as well as the overall amount of worms eaten, compared to the organisms' standard feeds.*

Target Species					Feeding Response Compared to Standard Feed		
	Scientific Name	**Common Name**	**Life Stage**	**Size**	**After First Time**	**After Multiple Times**	**Total Amount Eaten**
Ornamental Fishes	*Pethia conchonius*	Rosy Barb	juvenile/adult	2-3 cm	=	=	=
	Zebrasoma scopas	Scopus ang	adult	15-20 cm	↑	↑	↑
	Epalzeorhynchos frenatum	Rainbow Shark	fry	1 mm	=	=	=
	Amphiprion percula	Picasso Clownfish	adult	10-15 cm	=	=	↑
	Cirrhitidae	Hawkfish	adult	15-20 cm	=	=	↑
	Amphiprion ocellaris	Ocellaris Clownfish	juvenile/adult	1-8 cm	↑	↑	↑
	Amphiprion frenatus	Tomato Clownfish	juvenile/adult	1-8 cm	↑	↑	↑
	Premnas biaculeatus	Maroon Clownfish	juvenile/adult	1-10 cm	↑	↑	↑
	Pseudochromis fridmani	Orchid Dottyback	adult	6-8 cm	=	=	=
	Holacanthus tricolor	Rock Beauty Angelfish	adult	10-12 cm	=	=	=
	Halichoeres chrysus	Yellow Coris Wrasse	adult	6-8 cm	↑	↑	↑
	Poecilia latipinna	Sailfin Molly	adult	6-8 cm	↑	↑	↑
	Ecsenius bicolor	Bicolor Blenny	adult	6-8 cm	↑	↑	↑
	Betta spp.	Betta	adult	6-8 cm	↑	↑	↑
	Gambusia affinis	Mosquitofish	adult	6-8 cm	↑	↑	↑
	Cyprinodontiformes	Killifish	adult	6-8 cm	↑	↑	↑
	Hippocampus erectus	Lined Seahorse	adult	8-10 cm	↓	↓	↓
	Genicanthus bellus	Bellus Angelfish	adult	12 cm	=	=	↓
Other Fishes	*Acipenser fulvescens*	Lake Sturgeon	juvenile	8-13 cm	↑	↑	=
	Anoplopoma fimbria	Sablefish	early juvenile	0.5 g	↓	=	=
	Lutjanus campechanus	Northern Red Snapper	juvenile	8-13 cm	↓	=	=
	Sciaenops ocellatus	Red Drum	early juvenile	2 g	=	↑	↑
	Microgadus tomcod	Atlantic Tomcod	juvenile	8 cm	↓	↓	↓
	Sander vitreus	Walleye	fingerling	4-8 cm	↓	↓	↓
	Cyprinus carpio	Koi	adult	38-46 cm	↓	↓	↓
	Percina caprodes	Logperch	adult	10 cm	↑	=	=
	Paralichthys lethostigma	Southern Flounder	juvenile	1.5-20 cm	↓	=	=
	Oreochromis niloticus	Nile Tilapia	juvenile	3-8 cm	=	=	↑
	Menidia menidia	Atlantic Silverside	juvenile	5 cm	=	=	=
	Scaphirhynchus albus	Pallid Sturgeon	juvenile	8-13 cm	↑	↑	↓
	Pseudopleuronectes americanus	Winter Flounder	juvenile	6 cm	=	=	=
Birds	*Calidris alba*	Sanderling	adult	49-55 g	=	=	=
	Calidris minutilla	Least Sandpiper	adult	20-25 g	=	=	=
	Calidris pusilla	Semipalmated Sandpiper	adult	20-30 g	=	=	=

All target species ate the worms, however, for some species (e.g., Sablefish, Northern Red Snapper, Southern Flounder), repeated offerings of white worms were necessary to elicit a normal feeding response (Table 3.1). There were a few species (e.g.: Lined Seahorse, Atlantic Tomcod, Walleye, Koi) that did not eat as much as they typically did, even after repeated feedings of white worms. The majority of target species consumed white worms with the same intensity as they exhibit when offered their standard feeds (41%) or had a stronger feeding response to the white worms (35%). This latter group included sturgeons and ornamental fishes, specifically tang, clownfishes, wrasse, molly, blenny, betta, mosquitofish, and killifish (Table 3.1).

Most participants (56%) found using live white worms to be logistically on par with their current feed sources, or even easier to store and distribute (31%) than their standard feeds (Fig. 3.6). A small percentage (13%) found the live white worm samples to be more complicated or difficult to use compared to their current feed sources. All participants reported that there was no change in the water quality of the culture tanks from using live white worms.

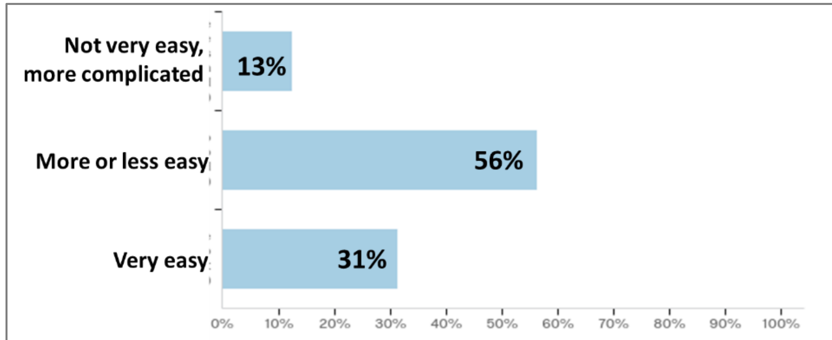


Figure 3.6. *Logistical experience of using live white worms compared to current feed sources.*

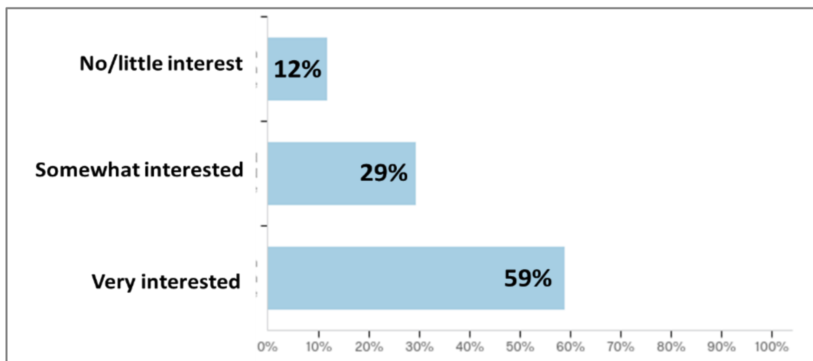


Figure 3.7. *Interest in using live white worms regularly if the product existed.*

When the participants were asked to assume if live white worms were available on a regular basis, what their likelihood of using the worms would be in their normal operations, the majority (88%) of respondents indicated that they were interested in using white worms with 59% very interested in using white worms (Fig. 3.7). Those who participated in the white worm testing, did so because they mostly were interested in finding a higher protein feed, diversifying their live feed options, and finding more suitable diets for the species being cultured (Fig. 3.8). Roughly one-third of respondents also were interested in lowering their live feed costs and utilizing diets low in fish meal and fish oil. Other reasons for participating in the white worm study included finding a live feed that would not impair water quality and finding a better live feed source for larval and juvenile fishes; aquaculturists who responded to this fed the white worms to Summer Flounder, Nile Tilapia, Sturgeon, Logperch, Rosy Barb, Rainbow Shark, Red Snapper, Walleye, Atlantic Tomcod, Atlantic Silverside, and Winter Flounder.

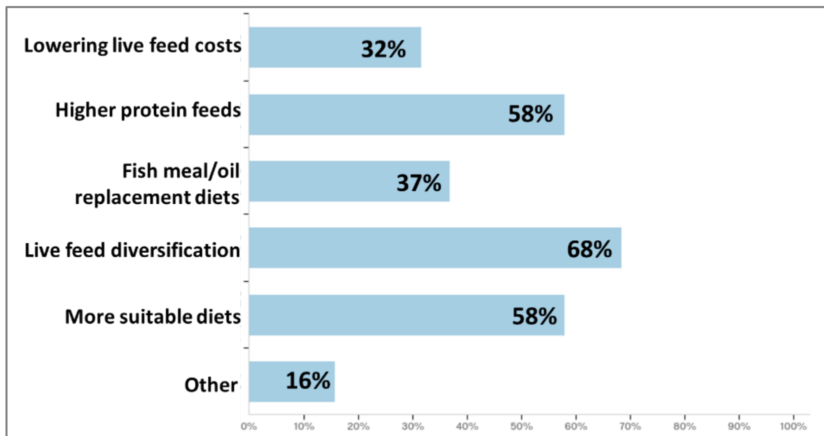


Figure 3.8. *Motivation of respondents for testing live white worms (multiple answers possible).*

Criteria that were deemed most important by the growers were condition of the white worms, ease of use, and year-round availability (Fig. 3.9). Price of the white worms and shipping frequency were rated somewhat important. Growers were less concerned with whether the worms would be certified organic and how they would be packaged. Two growers indicated in the 'other' category that how responsive the fish were to the worms and the nutritional profile of the worms were very important factors.

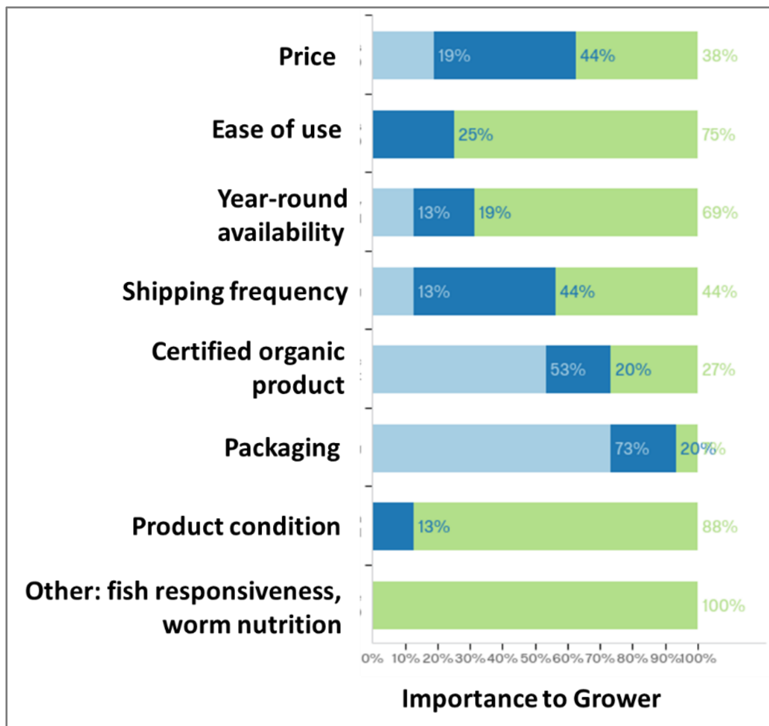


Figure 3.9. *Relative importance to growers of criteria related to live white worms. Light blue = not very important; dark blue = somewhat important; green = very important.*

Based on stakeholder input and the promising results of feeding white worms to ornamental fish, the best potential for using white worms is as a diet (live or possibly otherwise) for ornamental fishes. Ornamental culture is a growing sector in the aquaculture industry, valued at close to $30 million annually in Florida (DiMaggio, 2017). While protocols have been established to rear many of the 'typical' aquaria fishes like damsels, dottybacks, gobies, and blennies, there is a strong market demand for production of other fishes like tangs, wrasses, and butterflyfish (DiMaggio, 2017); for many of these latter species, feeding regimens have yet to be worked out. Judging from our experiences with live white worms, white worms may help with expanding the opportunities to culture these trickier species. Given that possibility, we asked the ornamental industry what they wanted nutritionally in a feed; the unanimous response was a live feed high in essential fatty acids, such as EPA and DHA.

OBJECTIVE 4: Evaluate the nutritional composition of white worms fed different enrichment products.

One of the limitations of using white worms as a feed is their relatively limited EPA and DHA content. We examined whether adding an enrichment high in fatty acids to their feed would result in white worms higher in n-3 LC-PUFAs while factoring in how cost effective these different feed strategies would be.

<u>Does adding an enrichment to white worm feed affect the fatty acid content of the worms?</u>

Methods:

Four white worm cultures, reared in plastic receptacles measuring 33 x 19 x 10 cm (6.4 L) and filled with sieved, seawater-dampened, organic, potting soil, were randomly chosen from the UNH stock cultures. These cultures had last been fed 3 weeks earlier. Three cultures were fed recently acquired spent brewing grains from Smuttynose Brewery (Portsmouth, NH) enriched with instant algae Reed Mariculture N-Rich High Pro Enrichment (75 mls mixed into 0.5 L grains; 1/3 cup mixture fed to each worm culture); each culture was harvested once after either 12, 24, or 48 hrs post feeding. A fourth worm culture was fed spent brewing grains only (not enriched), and was harvested 48 hrs later.

To gather sufficient sample volumes for analysis (~10 g worms), worms were harvested by placing each container on a heating pad until the worms began to congregate on the top of the soil away from the heat source. Worm aggregations were collected, transferred to seawater to remove adhering soil, drained, placed in labeled, plastic 2-dram vials, and held on dry ice until samples could be transferred to -80 °C storage. After all worm samples had been collected, they were packaged with dry ice and shipped overnight to New Jersey Feed Labs, LLC for compositional analysis.

White worms were analyzed to determine proximate and fatty acid composition. In this preliminary test, worms were not freeze dried and moisture content was not measured. Because replicate samples were

not collected in this preliminary trial, statistical analyses were not possible. However, proximate and fatty acid composition values for frozen white worms were compared to determine if any changes to the worms occurred by: 1) adding instant algae to the grains; and 2) varying the feeding duration prior to harvesting the worms.

Results:

Slight differences were observed in both proximate and fatty acid composition of white worms fed enriched and unenriched grains, and also between different feeding durations (12, 24, 48 hrs) prior to harvesting (Tables 4.1, 4.2). Maximum variation in white worm proximate composition between treatments was slight: protein = 1.52%, fat = 0.35%, and ash = 0.61% (Table 4.1).

Table 4.1. *Percent crude composition of frozen white worms after feeding on spent brewing grains for 48 hr (no enrichment), or enriched brewing grains for 12, 24, and 48 hr. Moisture content was not measured.*

	48 hr - no enrichment	12 hr	24 hr	48 hr
Protein (crude)	11.68	10.16	10.27	10.24
Fat (crude)	2.12	2.26	2.47	2.21
Ash	1.38	1.99	1.44	1.4

Variation also was observed in white worm fatty acid composition between treatments (Table 4.2). Of interest was the change in DHA from undetectable levels in worms fed only brewery grains to an increase of 1.31% of relative basis in worms fed grains enriched with instant algae and harvested 12 hr after feeding. DHA levels were present in worms fed the enriched grains with longer feeding durations too, but these levels decreased with time to 1.11% after 24 hr and 0.95% after 48 hr.

Table 4.2. *Fatty acid profile of frozen white worms after feeding on spent brewing grains for 48 hr (no enrichment), or enriched brewing grains for 12, 24, and 48 hr.*

	Duration	48 hr - no enrichment		12 hr		24 hr		48 hr	
		Relative	Sample	Relative	Sample	Relative	Sample	Relative	Sample
Fatty Acid Profile	C# : Dbl. Bonds	Basis %	Basis %	Basis %	Basis %	Basis %	Basis %	Basis %	Basis %
Caprylic	8:0	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Capric	10:0	0.28	0.00	0.26	0.00	0.23	0.00	0.20	0.00
Lauric	12:0	1.19	0.02	1.02	0.01	1.06	0.02	1.07	0.02
Myristic	14:0	5.72	0.10	5.41	0.03	5.44	0.10	5.16	0.09
Myristoleic	14:1	0.95	0.02	0.79	0.00	0.64	0.01	0.75	0.01
Pentadecanoic	15:0	0.47	0.01	0.65	0.00	0.46	0.01	0.48	0.01
Palmitic	16:0	5.21	0.09	8.52	0.05	6.71	0.13	5.90	0.11
Palmitoleic	16:1	3.05	0.05	2.63	0.01	2.80	0.05	2.92	0.05
Heptadecanoic	17:0	0.35	0.01	0.48	0.00	0.38	0.01	0.28	0.01
Stearic	18:0	3.21	0.06	3.70	0.02	2.85	0.05	2.84	0.05
Oleic	18:1ω9	2.78	0.05	6.83	0.04	3.93	0.07	3.56	0.06
Oleic	18:1ω7	1.02	0.02	1.42	0.01	1.29	0.02	1.28	0.02
Linoleic	18:2ω6	25.26	0.44	25.15	0.14	27.97	0.53	24.81	0.45
Linolenic	18:3ω3	2.58	0.04	2.49	0.01	2.69	0.05	2.34	0.04
Arachidic	20:0	0.18	0.00	0.22	0.00	0.25	0.00	0.20	0.00
Eicosanoic	20:1ω11	3.30	0.06	2.00	0.01	2.82	0.05	3.03	0.05
Eicosanoic	20:1ω9	0.35	0.01	0.56	0.00	0.49	0.01	0.43	0.01
Eicosadienoic	20:2ω6	8.49	0.15	6.95	0.04	7.71	0.15	8.10	0.15
Eicosatrienoic	20:3ω6	0.85	0.01	0.60	0.00	0.56	0.01	0.51	0.01
Eicosatrienoic	20:3ω3	0.44	0.01	0.27	0.00	0.31	0.01	0.30	0.01
Arachidonic	20:4ω6	8.72	0.15	5.65	0.03	5.96	0.11	6.72	0.12
Eicosapentaenoic (EPA)	20:5ω3	2.56	0.04	2.13	0.01	2.21	0.04	2.53	0.05
Docosapentaenoic	22:5ω6	0.00	0.00	0.36	0.00	0.41	0.01	0.38	0.01
Docosapentaenoic	22:5ω3	0.33	0.01	0.36	0.00	0.33	0.01	0.28	0.01
Docosahexaenoic (DHA)	22:6ω3	0.00	0.00	1.31	0.01	1.11	0.02	0.95	0.02
Lignoceric	24:0	0.00	0.00	0.20	0.00	0.26	0.00	0.00	0.00
Other	n/a	22.49	0.39	20.04	0.11	20.93	0.40	25.00	0.45
	Total	100.00	1.73	100.00	0.56	100.00	1.90	100.00	1.80
	Total % ω3	5.91	0.10	6.57	0.04	6.85	0.13	6.39	0.11
	Total % ω6	43.31	0.75	38.70	0.22	42.61	0.81	40.51	0.73

Implications:

From this very precursory study, it appears that white worms can metabolize enrichments like instant algae which can augment their fatty acid content, and in particular, elevate their DHA levels. In addition, it is likely that these nutritional benefits wane with increasing time post-feeding.

When should white worms be harvested to maximize the effects of an enrichment?

Methods:

An additional six white worm cultures were randomly chosen from the UNH stock cultures. These cultures had last been fed 2 weeks earlier. Three cultures were fed recently acquired spent brewing grains enriched with instant algae Reed Mariculture N-Rich High Pro Enrichment (75 mls mixed into 0.5 L grains; 1/3 cup mixture fed to each worm culture); each culture was harvested once after either 4, 6, 8, 10, or 12 hr post feeding. A sixth worm culture was fed spent brewing grains only (not enriched), and was harvested 4 hr later.

Worm samples (~10 g) from each culture were harvested and stored as per prior methods. After all worm samples had been collected, they were packaged with dry ice and shipped overnight to New Jersey Feed Labs, LLC for compositional analysis.

White worms were analyzed to determine proximate and fatty acid composition. Worms were not freeze dried but moisture content was measured. Proximate and fatty acid composition values for frozen white worms were compared to determine if changes to the worms occurred by reducing the feeding duration to <12 hr prior to harvesting the worms. Because replicate samples were not collected in this preliminary trial, statistical analyses were not possible.

Results:

Similar to the first preliminary enrichment trial, differences were observed in both proximate and fatty acid composition of white worms fed enriched and unenriched grains, and also between different feeding durations (4, 6, 8, 10, 12 hr) prior to harvesting (Tables 4.3, 4.4). Maximum variation in white worm proximate composition between treatments was slight: protein = 1.05%, fat = 0.33%, and ash = 0.13% (Table 4.3). Fat content was lowest in the worms fed unenriched grains but only differed by 0.03% from those worms harvested after 12 hr feeding duration. Based on only one replicate per treatment, a potential trend may exist of increased fat content with increased enrichment duration up to 10 hr (Table 4.3) and this also is reflected in the proportion of DHA relative to other fatty acids (Table 4.4).

Table 4.3. *Percent crude composition of frozen white worms after feeding on spent brewing grains for 4 hr (no enrichment), or enriched brewing grains for 4, 6, 8, 10, and 12 hr.*

	4 hr - no enrichment	4 hr	6 hr	8 hr	10 hr	12 hr
Moisture	87.29	87.14	86.95	86.58	86.34	87.46
Protein (crude)	7.38	7.50	7.45	7.98	7.78	6.93
Fat (crude)	1.86	1.93	1.99	2.01	2.19	1.89
Ash	0.93	0.89	0.89	0.89	0.98	0.85

Table 4.4. *Fatty acid profile of frozen white worms after feeding on spent brewing grains for 4 hr (no enrichment), or enriched brewing grains for 4, 6, 8, 10, and 12 hr.*

	Duration (hrs)	4 - no enrichment		4		6		8		10		12	
		Relative	Sample	Relative	Sample	Relative	Sample	Relative	Sample	Relative	Sample	Relative	Sample
Fatty Acid Profile	C# : Dbl. Bonds	Basis %	Basis %	Basis %	Basis %	Basis %	Basis %	Basis %	Basis %	Basis %	Basis %	Basis %	Basis %
Capric	10:0	0.21	0.003	0.27	0.004	0.22	0.003	0.19	0.003	0.27	0.004	0.25	0.004
Lauric	12:0	1.11	0.015	1.30	0.018	1.21	0.017	1.14	0.016	1.27	0.020	1.42	0.022
Myristic	14:0	5.75	0.078	5.77	0.081	5.85	0.080	5.34	0.073	5.90	0.092	5.95	0.094
Myristoleic	14:1	0.75	0.010	0.78	0.011	0.83	0.011	0.76	0.010	0.80	0.013	0.78	0.012
Pentadecanoic	15:0	0.41	0.006	0.43	0.006	0.47	0.006	0.43	0.006	0.46	0.007	0.46	0.007
Palmitic	16:0	6.24	0.085	5.86	0.082	5.88	0.081	5.76	0.079	6.39	0.100	5.95	0.094
Palmitoleic	16:1	3.25	0.044	3.28	0.046	3.24	0.045	2.68	0.037	3.21	0.050	3.12	0.050
Heptadecanoic	17:0	0.25	0.003	0.25	0.004	0.32	0.004	0.21	0.003	0.32	0.005	0.35	0.006
Stearic	18:0	2.72	0.037	2.82	0.040	2.72	0.037	2.88	0.039	2.86	0.045	2.75	0.044
Oleic	18:1ω9	3.70	0.050	3.25	0.046	3.26	0.045	3.61	0.049	3.78	0.059	3.68	0.058
Oleic	18:1ω7	1.07	0.015	0.94	0.013	1.05	0.014	0.93	0.013	1.16	0.018	1.05	0.017
Linoleic	18:2ω6	26.45	0.361	25.63	0.361	26.04	0.358	26.51	0.362	25.39	0.396	26.18	0.416
Linolenic	18:3ω3	2.52	0.034	2.70	0.038	2.56	0.035	2.72	0.037	2.51	0.039	2.46	0.039
Octadecatetraenoic	18:4ω3	0.00	0.000	0.00	0.000	0.17	0.002	0.00	0.000	0.00	0.000	0.00	0.000
Arachidic	20:0	0.21	0.003	0.21	0.003	0.19	0.003	0.23	0.003	0.21	0.003	0.17	0.003
Eicosanoic	20:1ω11	3.18	0.043	2.89	0.041	2.75	0.038	2.87	0.039	2.82	0.044	2.61	0.041
Eicosanoic	20:1ω9	0.66	0.009	0.44	0.006	0.39	0.005	0.41	0.006	0.44	0.007	0.44	0.007
Eicosadienoic	20:2ω6	8.38	0.114	8.44	0.119	8.03	0.110	8.17	0.112	7.80	0.122	8.08	0.128
Eicosatrienoic	20:3ω6	0.70	0.010	0.89	0.012	0.74	0.010	0.58	0.008	0.68	0.011	0.79	0.013
Eicosatrienoic	20:3ω3	0.41	0.006	0.38	0.005	0.36	0.005	0.47	0.006	0.34	0.005	0.42	0.007
Arachidonic	20:4ω6	7.51	0.102	7.31	0.103	7.26	0.100	7.09	0.097	6.91	0.108	6.85	0.109
Eicosapentaenoic (EPA)	20:5ω3	2.31	0.031	2.38	0.033	2.47	0.034	2.75	0.038	2.58	0.040	2.41	0.038
Erucic	22:1ω11	0.34	0.005	0.30	0.004	0.32	0.004	0.25	0.003	0.32	0.005	0.32	0.005
Docosapentaenoic	22:5ω6	0.0	0.0	0.19	0.003	0.20	0.003	0.00	0.000	0.29	0.005	0.17	0.003
Docosapentaenoic	22:5ω3	0.41	0.006	0.38	0.005	0.42	0.006	0.43	0.006	0.46	0.007	0.46	0.007
Docosapentaenoic (DHA)	22:6ω3	0.00	0.000	0.69	0.010	0.77	0.011	0.71	0.010	1.06	0.017	0.84	0.013
Other	n/a	21.46	0.293	22.23	0.313	22.27	0.306	22.88	0.313	21.75	0.339	22.05	0.350
	Total	100.00	1.364	100.00	1.407	100.00	1.374	100.00	1.366	100.00	1.559	100.00	1.588
	Total % ω3	5.65	0.077	6.54	0.092	6.76	0.093	7.31	0.100	7.16	0.112	6.59	0.105
	Total % ω6	43.03	0.587	42.46	0.597	42.28	0.581	42.35	0.578	41.07	0.640	42.07	0.668

Implications:

This second preliminary trial corroborates the first trial by also showing that white worms can metabolize enrichments like the instant algae which can augment their fatty acid content, and in particular, elevate their DHA levels. Here we looked at the effects of adding an enrichment to the worm feed (brewery grains) and harvesting the worms within 12 hr after feeding. Like the first trial, it appears that these nutritional benefits are affecting by time post-feeding with worms requiring a sufficient time to feed and metabolize the enrichment. It seems DHA levels in the worms is highest when harvested after 10 hr post-feeding.

<u>Which enrichment yields the highest fatty acid content in white worms?</u>

Methods:

A common garden experiment was designed to assess the nutrient composition of white worms fed spent brewing grains enriched with various additives containing purportedly high levels of omega-3 fatty acids at the UNH Coastal Marine Laboratory. Five feed enrichments (Reed Mariculture N-Rich High Pro Enrichment [instant algae], UltraCruz Pure Salmon Oil for Dogs [salmon oil], UltraCruz Equine Pure Flax Oil [flax oil], Bob's Red Mill Premium Whole Ground Flaxseed Meal [flaxseed meal], and Bob's Red Mill

Wheat Bran [wheat bran]) plus a treatment containing no enrichment [grains] (e.g., just spent brewing grains), replicated in triplicate, were evaluated in white worm cultures held at ambient temperatures (Table 4.5). The white worm cultures were randomly chosen from the UNH stock cultures which had last been fed 1 week earlier.

Worm cultures (n=15 worm cultures) were fed recently acquired spent brewing grains enriched with one of the five enrichment treatments. For liquid enrichments (instant algae, salmon oil, and flax oil), 75 mls was mixed into 0.5 L grains. For solid enrichments (flaxseed meal and wheat bran), 0.5-1 c was mixed into 0.5 L grains or until the same consistency was reached as the liquid-enriched grains. Each worm culture was fed 1/3 c of the mixture. A sixth treatment (n=3 worm cultures) was not enriched and fed grains only. All worm cultures were harvested after 10 hrs.

Table 4.5. *Characteristics of products tested as enrichments to white worm feed (spent brewery grains) and the resulting effects to white worm EPA and DHA.*

Enrichment Treatment	Dose Amount	Worm Sample EPA* (% dry matter)	Worm Sample DHA* (% dry matter)	Shelf Life (months)	Bulk Amount	Total Doses	Total Factor Cost	Unit Cost	Average Product Cost per Unit of Increased EPA	Average Product Cost per Unit of Increased DHA
Instant algae	75 mL	0.28b	0.22a	4	0.95 L	12.7	$56.15	$4.42	$7.02	$2.55
Salmon oil	75 mL (33.5 g)	0.48a	0.23a	4-10	907 g	27.1	$21.00	$0.77	$0.75	$0.91
Flax oil	75 mL	0.20b	0.01b	12	3.8 L	50.7	$38.00	$0.75	n/a	$0.03
Flaxseed meal	113 g (1 cup)	0.23b	0.00b	6	453 g	4.0	$3.39	$0.85	n/a	$38.00
Wheat bran	55.5 g (1 cup)	0.25b	0.00b	12	227 g	4.1	$1.69	$0.41	$1.13	n/a
Grains	0 mL	0.20b	0.00b	n/a	n/a		$0	$0		

*Differing letters within a column denote significant differences ($p<0.05$) between enrichments.

Nutritional analysis:

Duplicate 10 g samples of each feed treatment and worm samples (~10 g) from each culture were harvested, snap frozen on dry ice, and stored in -80 °C storage until analyses. After all samples had been collected, they were freeze dried for 48 hr, then packaged with dry ice and shipped overnight to New Jersey Feed Labs, LLC for proximate and fatty acid composition analysis.

Mean proximate and fatty acid composition values for feeds were compared using Kruskal-Wallis followed by a Dunn Test with an adjusted p-value. White worms were compared using one-way ANOVA followed by Tukey's HSD pairwise comparison tests (R version 3.2.1.).

Although the response variables reflected data collected or calculated from many pooled worms collected from an individual culture container, replicate culture containers were considered

experimental units (N = 3) for all statistical analysis. All effects/differences were considered significant at $P < 0.05$.

Economic analysis:

To calculate the unit cost of using each enrichment product, the total factor cost (i.e., shelf price of the enrichment) was divided by the number of total doses (or bulk amount/dose amount).

The average product cost gauges which enrichment will boost these nutrients at the least cost per EPA and DHA percent composition in the final product (white worms), or, in other words, how can white worms be grown to produce the highest levels of targeted nutrients in the most cost-effective way.

To calculate average product cost of EPA concentration per 0.5 L of grain feed, the total cost of the supplement was divided by the change in percent composition of EPA:

$$TFC_{EPA} / [N_{EPA2} - N_{EPA1}]$$

where:

TFC_{EPA} is the total factor cost of the supplement per 0.5 L of grain.

N_{EPA2} is the percent concentration of EPA in white worms that were fed grains with the supplement in question.

N_{EPA1} is the percent concentration of EPA in white worms fed grains without that supplement.

These calculations were applied to each supplement and calculated for both EPA and DHA, as shown in Table 4.5.

Results:

Feeds varied as a result of the enrichment product added with protein ($p<0.001$), fat ($P<0.001$), and ash ($p<0.001$) all changing significantly (Table 4.6). Protein was highest (22-23%) in unenriched grain or grain enriched with flaxseed meal, and lowest (10%) in grains enriched with flaxseed oil and salmon oil. The inverse relationship was observed for fat content: flaxseed oil or salmon oil added to grains yielded the highest fat content at 2% compared to <1% in unenriched grain or grain + wheat bran. Ash content ranged from 1% to 9% and was highest in grain + instant algae and lowest in grain + flaxseed oil or salmon oil.

Proximate composition of white worms was mostly unaffected by feed enrichment (Table 4.6). There were no differences in either the protein ($p=0.538$) or fat ($p=0.258$) content of the worms fed any of the enriched diets. Worm composition was 55-59% protein and 15-17% fat. Only ash content ($p=0.002$) was affected by the enrichments with flaxseed meal (5.2%) and salmon oil (5.3%) having lower ash than either grain (6.3%) or flaxseed oil (6.2%), but neither grouping differing significantly from instant algae (5.7%) or wheat bran (5.6%).

Significant differences in feed fatty acid composition were detected in all fatty acids analyzed between different enrichment treatments (Table 4.6). Worm fatty acid composition also was significantly affected by feed enrichment treatment in most cases (Table 4.6). Of particular interest, worms fed grain enriched with salmon oil had the highest EPA content (sample content = 0.48%, relative content = 3.91%) compared to worms fed the other enrichments (sample content range = 0.20-0.28%; relative content = 1.84-2.47%; Table 4.6). DHA content was highest in worms fed grains enriched with either salmon oil (sample content = 0.23%, relative content = 1.83%) or instant algae (sample content = 0.22%, relative content = 1.94%), and compared to worms fed all remaining enrichment treatments enrichments (sample content range = 0-0.01%; relative content = 0-0.06%; Table 4.6).

Table 4.6. *Proximate, EPA, and DHA composition of spent brewing grain; grain enriched with flaxseed oil, flaxseed meal, salmon oil, instant algae, or wheat bran; and white worms fed these feeds. Least-square means and P-values are provided for both feed and worms. For parameters exhibiting significant enrichment treatment effects, means with different letter labels are significantly different (P < 0.05).*

			Enrichments					
Parameter	Component	Grain	Flaxseed Oil	Flaxseed Meal	Salmon oil	Instant Algae	Wheat bran	P-values
Protein	Feed	21.93a	10.31c	22.63a	10.43c	20.16b	19.69b	<0.001
(% dry matter)	Worms	55.87	55.15	58.72	56.23	55.09	55.04	0.538
Fat	Feed	6.67d	55.9a	24.69b	56.7a	9.07c	5.88d	<0.001
(% dry matter)	Worms	14.56	15.82	16.1	16.94	15.36	14.96	0.258
Ash	Feed	3.31b	1.67c	3.27b	1.49c	8.75a	3.56b	<0.001
(% dry matter)	Worms	6.3a	6.17a	5.18b	5.27b	5.68ab	5.55ab	0.002
Relative EPA	Feed	0bc	0bc	0bc	8.25a	1.46b	0.40c	<0.001
(% dry matter)	Worms	2.01b	1.84b	2.05b	3.91a	2.43b	2.47b	<0.001
Sample EPA	Feed	0a	0a	0a	4.05a	0.09b	0.02b	<0.001
(% dry matter)	Worms	0.20b	0.20b	0.23b	0.48a	0.28b	0.25b	<0.001
Relative DHA	Feed	0c	0c	0c	8.19a	6.68b	0.39c	<0.001
(% dry matter)	Worms	0b	0.06b	0b	1.83a	1.94a	0b	<0.001
Sample DHA	Feed	0c	0c	0c	4.03a	0.43b	0.02c	<0.001
(% dry matter)	Worms	0b	0.01b	0b	0.23a	0.22a	0b	<0.001

The total factor costs of the supplements per 0.5 L of grain feed varied from $1.69 for wheat bran to $56.15 for instant algae (Table 4.5). While these costs are helpful in determining which supplement is the best choice to improve the nutritional content of the worms, calculating the average product cost is key because it determines the most cost-effective method for increasing a specific nutrient. Wheat bran had the least cost per percent increase of EPA ($0.01 per % EPA), which was expected given the low price of the supplement, however it may not be an optimal supplement; the increase in EPA from wheat

bran was marginal, and it did not result in any increase in DHA. Salmon oil, on the other hand, had the second least expensive cost per % increase in EPA at $0.75 per % increase, followed by flax seed ($1.13), and instant algae ($7.02). In addition, salmon oil had the least cost per percent of DHA content. It cost $0.91 per percent increase in DHA for salmon oil, as compared to $2.55, and $38.00 for instant algae and flax oil, respectively.

Implications:

Although both flaxseed and salmon oils increased the fat content in the spent brewing grains, only the salmon oil led to greater EPA content in the worms. More importantly, salmon oil enriched grains also resulted in worms high in DHA. In addition to salmon oil, grains enriched with instant algae yielded worms with equally high DHA content. However, we recommend using salmon oil over instant algae as a more cost-effective enrichment because:

1) Salmon oil has a longer shelf life if refrigerated (up to 10 months) as opposed to instant algae which must be refrigerated but only lasts 4 months, and
2) Salmon oil also costs less per percent of combined increase in EPA ($0.75) and DHA ($0.91). This is the least costly method we tested to achieve increases in these fats (Table 4.5).

How much enrichment should be added to the feed?

Based on the previous experiment, salmon oil was chosen as the most cost-effective enrichment in terms of lower price, longer shelf life, and resultant high levels of DHA and EPA in the white worms compared to the other enrichments considered. To determine if varying the amount of salmon oil added to grains would affect white worm composition, an experiment evaluating three dosage levels (low, medium, high) was conducted.

Methods:

Nine white worm cultures (3 dosage treatments x 3 replicates) were randomly chosen from the UNH stock cultures which had last been fed 1 week earlier. Worm cultures were fed 3/4 c of a blend of recently acquired spent brewing grains (0.5 L) enriched with one of the three amounts of salmon oil: 75 mls [low], 150 [medium], 225 mls [high]. All worm cultures were harvested after 12 hr.

Nutritional analysis:

Triplicate samples of each feed treatment and worm samples (~10 g) from each culture were harvested, snap frozen on dry ice, and stored in -80 °C storage until analyses. After all samples had been collected, they were freeze dried for 48 hr, then packaged with dry ice and shipped overnight to New Jersey Feed Labs, LLC for proximate and fatty acid composition analysis.

Mean proximate and fatty acid composition values for both feeds and white worms were compared using one-way ANOVA followed by Tukey's HSD pairwise comparison tests (R version 3.2.1).

Although the response variables reflected data collected or calculated from many pooled worms collected from an individual culture container, replicate culture containers were considered experimental units ($N = 3$) for all statistical analysis. All effects/differences were considered significant at $P < 0.05$.

Economic analysis:

To calculate the unit cost of using each enrichment product, the total factor cost (i.e., shelf price of the enrichment) was divided by the number of total doses (or bulk amount/dose amount).

The average product cost gauges which enrichment will boost these nutrients at the least cost per EPA and DHA percent composition in the final product (white worms), or, in other words, how can white worms be grown to produce the highest levels of targeted nutrients in the most cost-effective way.

To calculate average product cost of EPA concentration per 0.5 L of grain feed, the total cost of the supplement was divided by the change in percent composition of EPA:

$$TFC_{EPA} / [N_{EPA2} - N_{EPA1}]$$

where:

TFC_{EPA} is the total factor cost of the supplement per 0.5 L of grain.

N_{EPA2} is the percent concentration of EPA in white worms that were fed grains with the supplement in question.

N_{EPA1} is the percent concentration of EPA in white worms fed grains without that supplement.

These calculations were applied to each supplement and calculated for both EPA and DHA, as shown in Table 4.8.

Results:

The amount of salmon oil added to the grains affected the proximate composition of the feed ($p<0.001$; Table 4.7). Both protein and ash decreased with increasing salmon oil dosage, while the opposite trend occurred with fat.

Table 4.7. *Proximate and fatty acid composition (% fatty acid methyl esters[FAMEs]) of white worms and feeds enriched with low, medium, and high salmon oil doses. Only fatty acids representing >1% of total FAMEs in at least one treatment group or those of special interest are reported individually. Least-square means and P-values are provided for both feed and worms. For parameters exhibiting significant salmon oil dosage treatment effects, means with different letter labels are significantly different (P < 0.05).*

		Enrichment Dosage			
Parameter	Component	Low	Medium	High	P-value
Protein (% dry matter)	Feed	12.95a	10.27b	7.63c	<0.001
	Worms	57.77a	55.39ab	53.37b	0.05
Fat (% dry matter)	Feed	48.38c	61.89b	70.47a	<0.001
	Worms	18.07	19.94	22.67	0.085
Ash (% dry matter)	Feed	1.81a	1.27b	0.98b	<0.001
	Worms	5.51	6.69	5.75	0.22
Relative EPA (% dry matter)	Feed	8.1	8.28	8.33	0.35

	Worms	3.10b	3.84ab	4.66a	0.044
Sample EPA (% dry matter)	Feed	3.54c	4.62b	5.24a	<0.001
	Worms	0.40b	0.58ab	0.83a	0.037
Relative DHA (% dry matter)	Feed	7.99b	8.19ab	8.37a	0.043
	Worms	1.35b	2.38ab	3.46a	0.027
Sample DHA (% dry matter)	Feed	3.49c	4.57b	5.26a	<0.001
	Worms	0.17b	0.37ab	0.61a	0.024
Relative Myristic 14:0	Feed	0.39b	4.84a	4.89a	<0.001
	Worm	5.38	5.73	5.86	0.628
Sample Myristic 14:0	Feed	0.17c	2.70b	3.08a	<0.001
	Worm	0.7	0.86	1.04	0.13
Relative Palmitic 16:0	Feed	14.24a	14.01b	13.91b	0.001
	Worm	6.87	7.5	7.89	0.216
Sample Palmitic 16:0	Feed	6.22c	7.81b	8.76a	<0.001
	Worm	0.89	1.13	1.4	0.076
Relative Stearic 18:0	Feed	2.32	2.38	2.39	0.093
	Worm	2.67a	2.58ab	2.43b	0.049
Sample Stearic 18:0	Feed	1.01c	1.33b	1.51a	<0.001
	Worm	0.35	0.38	0.43	0.093
Relative Myristoleic 14:1	Feed	0.39	0.37	0.37	0.696
	Worm	0.7	0.59	0.55	0.063
Sample Myristoleic 14:1	Feed	0.17b	0.21ab	0.23a	0.02
	Worm	0.09	0.09	0.1	0.62
Relative Palmitoleic 16:1	Feed	5.57b	5.81a	5.85a	0.002
	Worm	3.27	3.85	4.2	0.97
Sample Palmitoleic 16:1	Feed	2.43c	3.24b	3.68a	<0.001
	Worm	0.43	0.58	0.74	0.655
Relative Oleic 18:1W9	Feed	13.91b	14.06ab	14.18a	0.024
	Worm	4.82b	6.03ab	7.19a	0.035
Sample Oleic 18:1W9	Feed	6.07c	7.85b	8.92a	<0.001
	Worm	0.63b	0.92ab	1.28a	0.039
Relative Oleic 18:1W7	Feed	3.18b	3.28ab	3.31a	0.028
	Worm	1.43	1.7	1.83	0.071
Sample Oleic 18:1W7	Feed	1.39c	1.83b	2.08a	<0.001
	Worm	0.19b	0.26ab	0.33a	0.058
Relative Eicosanoic 20:1W9	Feed	2.88b	2.96b	3.06a	0.002
	Worm	0.77b	1.08ab	1.28a	0.046
Sample Eicosanoic 20:1W9	Feed	1.26c	1.65b	1.93a	<0.001
	Worm	0.10b	0.16ab	0.23a	0.036
Relative Eicosanoic 20:3W6	Feed	0.12	0.15	0.13	0.25
	Worm	0.48	0.51	0.4	0.497
Sample Eicosanoic 20:3W6	Feed	0.05b	0.08a	0.06a	0.005
	Worm	0.06	0.07	0.07	0.515

Relative Erucic 22:1W11	Feed	7.97b	8.42a	8.64a	0.009
	Worm	1.25b	2.13ab	2.95a	0.027
Sample Erucic 22:1W11	Feed	3.48c	4.70b	5.44a	<0.001
	Worm	0.16b	0.33ab	0.52a	0.02
Relative Linoleic 18:2W6	Feed	5.40a	3.73b	2.92c	<0.001
	Worm	23.11	22.79	21.04	0.672
Sample Linoleic 18:2W6	Feed	2.36a	2.07b	1.84c	0.001
	Worm	3.01	3.34	3.71	0.193
Relative Linoleic 18:3W3	Feed	1.30a	1.12b	1.08b	0.005
	Worm	4.45	2.38	2.3	0.341
Sample Linoleic 18:3W3	Feed	0.57c	0.62b	0.68a	0.001
	Worm	0.58	0.35	0.41	0.527
Relative Arachidonic 20:4W6	Feed	0.56	0.54	0.56	0.492
	Worm	5.55a	4.62ab	3.75b	0.025
Sample Arachidonic 20:4W6	Feed	0.25c	0.30b	0.35a	0.001
	Worm	0.72a	0.67b	0.66b	0.014
Relative Docosapentaenoic 22:5W3	Feed	0.40b	1.79a	0.45b	<0.001
	Worm	0.64b	0.84ab	0.96a	0.018
Sample Docosapentaenoic 22:5W3	Feed	0.17c	0.10a	0.28b	<0.001
	Worm	0.35	0.17	0.13	0.594

Proximate composition of white worms was mostly unaffected by enrichment concentration (Table 4.7). There were no differences in either the fat (mean: 18-23% dry matter; p=0.085) or ash (mean: 5.5-6.7% dry matter; p=0.22) content of the worms fed any of the salmon oil dosages. Only the protein content (p=0.05) was affected by the enrichment treatment with protein content decreasing with increasing salmon oil dosage (means: low=58%, medium=55%, high=53% dry matter), though the medium dosage worms did not statistically vary from either the low or high dosage worms (Table 4.7).

Feed fatty acid composition was strongly affected by salmon oil dosage. All 16 fatty acids analyzed exhibited differences in the feeds due to the enrichment amount (Table 4.7). In most cases, increasing salmon oil dosage yielded higher amounts of the fatty acid. On the other hand, only half (n=8) of the fatty acids analyzed in the worms were significantly affected by the enrichment dosage: EPA, DHA, 18:0, 18:1w9, 20:1w9, 22:1w11, 20:4w6, and 22:5w3 (Table 4.7). In all cases except for stearic (18:0) and arachidonic (20:4w6) acids, worm fatty acid concentration varied significantly as follows: low dosage ≤ medium dosage ≤ high dosage.

The total factor costs of adding salmon oil per 0.5 L of grain feed varied from $0.77 for a low dosage to $2.33 for a high dosage (Table 4.8). While these costs are helpful in determining which dosage of salmon oil is the best choice to improve the nutritional content of the worms, calculating the average product cost is key because it determines the most cost-effective method for increasing a specific nutrient. In contrast to total factor costs, a high dosage of salmon oil had the least cost per percent increase of EPA ($0.33 per % EPA) compared to the low dosage ($1.05 per % EPA). Similarly, the high dosage resulted in the least cost percent increase of DHA ($0.34 per % DHA).

Table 4.8. *Nutritional and economic impact of using varying amounts of salmon oil as an enrichment to spent brewing grains for white worm feed.*

Salmon Oil Dosage	Dose Amount	Worm Sample EPA* (% dry matter)	Worm Sample DHA* (% dry matter	Shelf Life (months)	Bulk Amount	Total Doses	Total Factor Cost	Unit Cost	Average Product Cost per Unit of Increased EPA	Average Product Cost per Unit of Increased DHA
Low	75 mL (33.5 g)	0.40b	0.17b	4-10	907 g	27.1	$21.00	$0.77	$1.05	$1.24
Medium	150 mL (67 g)	0.58ab	0.37ab	4-10	907 g	13.5	$21.00	$1.56	$0.55	$0.57
High	225 mL (100.5 g)	0.83a	0.61a	4-10	907 g	9.0	$21.00	$2.33	$0.33	$0.34
None	0 mL (0 g)	0.2	0.0	n/a	n/a		$0	$0		

*Differing letters within a column denote significant differences ($p<0.05$) between dosages.

Implications:

When factoring in the effect of the three salmon oil dosages on the fatty acid composition of the white worms, and in particular the amount of EPA and DHA, a high dosage of salmon oil is the most cost-effective enrichment we tested (Table 4.8). Administering higher doses of salmon oil resulted in the largest increased of both EPA and DHA, further reducing the cost per percent of EPA and DHA concentrations found in Table 4.5. The cost per increase in EPA was reduced to $0.33 per 0.5 L of grain feed. The cost of percent DHA in worms per 0.5 L grain feed was $0.34.

Overall Conclusions

Upon fish culturists' interests in finding an alternate live feed high in high in essential fatty acids, such as EPA and DHA, we investigated whether we could alter the fatty acid content of live white worms through dietary supplements. We evaluated the effects of five different easily available supplements added to standard white worm feed (spent brewing grains). The supplements included instant algae, salmon oil, flax oil, flaxseed meal, and wheat bran.

Because the costs of the supplements varied, it was useful to calculate the average product cost to see how much each supplement cost per unit increase of nutrient concentration in white worms. Wheat bran was the least expensive way to increase EPA levels, but the increase was marginal and supplementing with wheat bran did not increase DHA in our sample. Salmon oil was the most cost-effective means of increasing DHA, and the second-most effective way to increase EPA. The combined results make salmon oil the most ideal supplement out of the ones tested. Our results also show that a high dose of salmon oil fed to white worms shortly before harvest is the most efficient means of increasing EPA and DHA levels and the nutritional value of white worms.

OBJECTIVE 5: Improve white worm production potential.

The main bottleneck in scaling up white worm production is our current harvesting system. Currently, worms are harvested by a very rudimentary heating process whereby the worm culture containers are heated from below by electric heating pads, waiting for several hours for the soil to reach a high enough temperature that causes the worms to move to the soil surface, and then gently and carefully removed by hand aggregated worms and transferring them into clean vessels with forceps. This process can take hours to harvest relatively small amounts of worms, and may adversely affect the unharvested juvenile worms and cocoons if the soil remains too hot for too long. This process is slow, inefficient, and laborious. In addition, with this process, it is not possible to harvest a worm culture completely so determining total worm biomass/culture can only be estimated. Because harvesting the worms effectively is so critical to the success of a white worm aquaculture project, I teamed up with UNH engineers Drs. Ken Baldwin and Barbaros Celikkol, and together, we are mentoring Andrew Pompeo, an Ocean Engineering Master's student. At this point in time, Andrew has completed his harvesting experiments and is in the process of writing his thesis (expected graduation of 12/17).

Heat transfer in soil:

Before designing a harvesting system, we had to understand how heat transferred through the soil. In particular, we were concerned with how deep the soil could be to efficiently harvest worms, while keeping the temperature in the soil from rising to a temperature that would harm the larvae and eggs. White worm eggs can withstand a temperature of 30 °C for 30 min before they begin to die (Ivleva 1973). At 25 °C, worms will start to migrate away from the heat source. Our goal was to have the soil reach a minimum of 25 °C and maximum of 30 °C. To figure this out, Andrew built a model of a guarded hot plate in SolidWorks software and used the Thermal Analysis Simulation to model the temperature at different locations in the soil. The soil depth was set to 10 cm deep.

The results from the SolidWorks model indicated that a much thinner layer of soil – 4 cm - would be necessary to reach a harvesting temperature. Different thermal conductivity values were used, but testing with actual soil was needed to understand exactly how the heat travels through the soil (i.e., worm cultures). Andrew designed an experiment and recorded the soil temperature with 6 temperature probes at 2 cm depth intervals from the surface to 10 cm deep of soil where a heat source (heating pad) was located (Fig. 5.1).

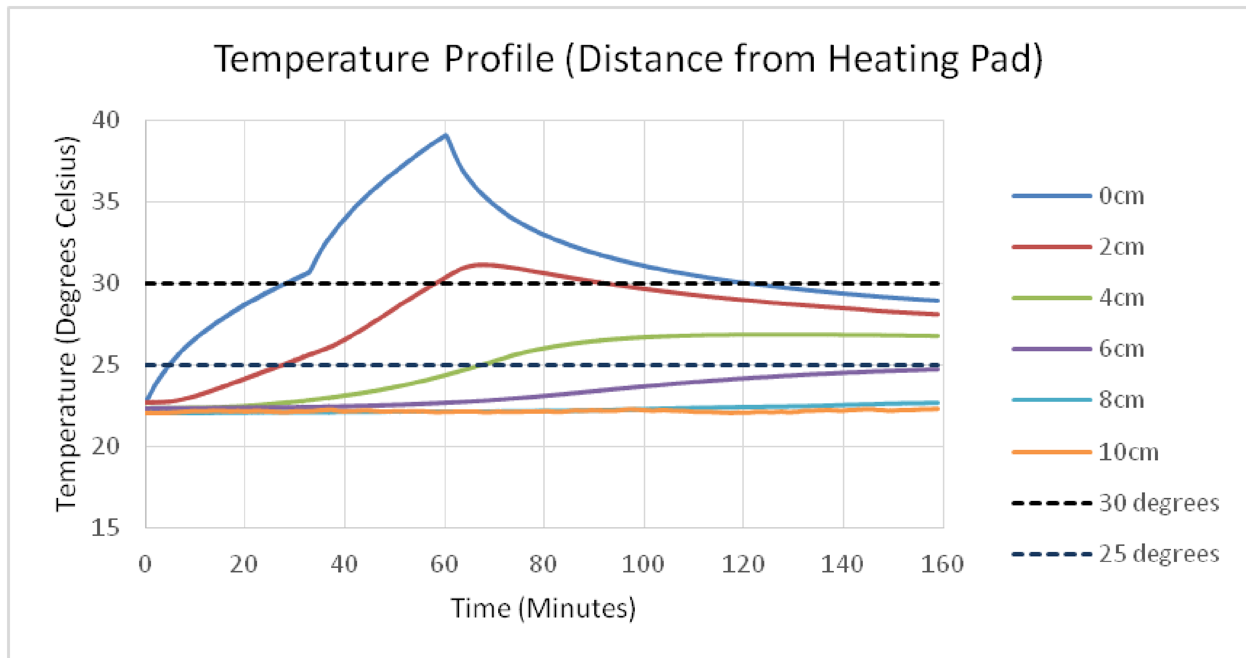


Figure 5.1. *Temperature versus time measured by each temperature probe at each 2cm interval in the soil. The temperature probe laid directly on top of the heating pad is represented by 0cm; 10cm represents the temperature probe exposed to the surface.*

Soil layers 0-4 cm reached at least 25 °C, which means, theoretically, these are the layers from which worms will migrate. Soil layers 6-10 cm will collect worms as the temperature in these layers does not exceed 25 °C. Based on these results, a similar soil experiment containing only 4 cm of soil was conducted, with three probes measuring temperature on the surface of the heating pad (beneath 4 cm soil) and three temperature probes at the surface (on top of the soil; Fig. 5.2). Based on the 4 cm depth temperature study (Fig. 5.2), we decided that a 2 cm layer of soil would be better suited for harvesting since the temperature of soil 4 cm away from the heating pad did not exceed 25 °C.

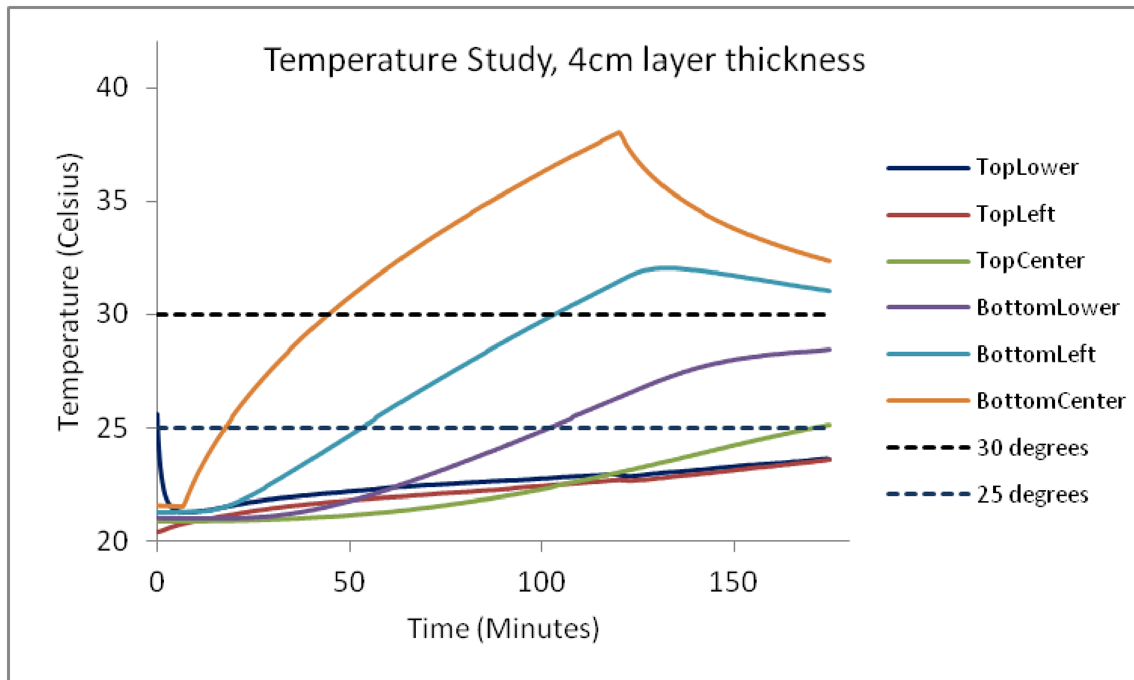


Figure 5.2. *Temperature versus time measured by three temperature probes at the surface and bottom of 4 cm of soil. The bottom temperature probes were laid directly on top of the heating pad; the top temperature probes were exposed to the surface.*

Harvester design and construction:

The next steps we took were the design, construction, and testing of a prototype harvester. We chose from three previously proposed designs. The premise of the design we selected projected heat (heating pad) downward through the soil, forcing the worms out of the soil and through a 2.5 mm screen at the bottom to a "clean" area (Fig. 5.3). The soil was contained by wooden sidewalls that also acted as insulation to retain the heat (Foam insulation and an aluminum sheet were added as extra modifications to retain heat.). A weight, placed on top of the heating pad, provided a seal to prevent heat loss. A Plexiglas sheet was placed below the harvester to collect the fallen worms.

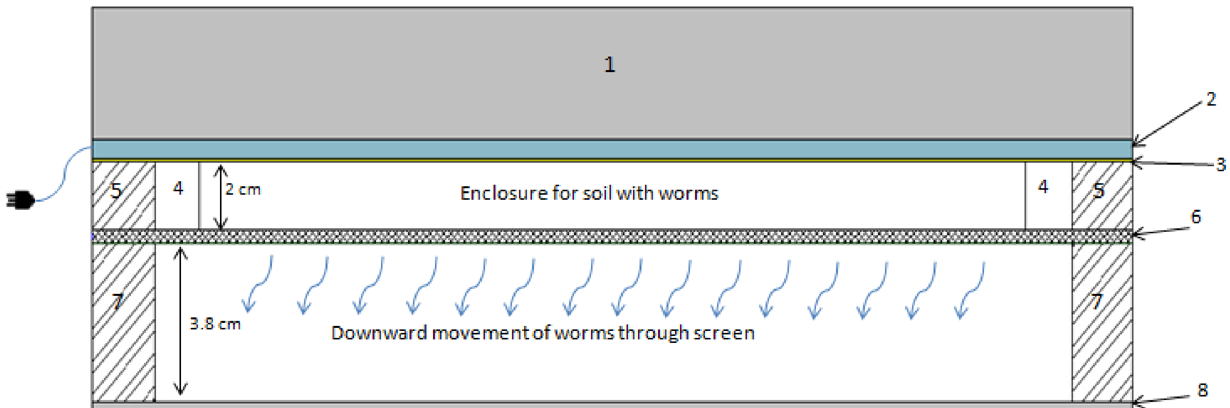


Figure 5.3. *Cross section of prototype harvester. The numbered labels correlate to different components of the harvester. 1- Weight, 2- Heating Pad, 3- Aluminum Sheet, 4- Insulation, 5- Wooden Side Walls, 6- Screen, 7- Wooden Supports, 8- Plexiglass sheet.*

A series of screen sizes (0.5 mm, 2.5 mm, and 3.2 mm) was evaluated to determine the proper screen size to minimize soil but allow worms to pass through the screen. Sieves of the varying screen sizes were used as experimental harvesters. Worms and 5 cm depth of soil were added into the sieve, and a heating pad was placed on top of the soil. The heating pad was turned on and reset every hour for three hours. At the end of three hours, observations were made on the quantity of worms that were tangled in the screen versus moved through the screen, and the quantity of soil that fell through the screen. Using a 0.5 mm screen resulted in more worms getting tangled in the screen and sometimes dying from the heat compared to the larger screens (Fig. 5.4). The 3.2 mm screen allowed more soil to fall through compared to the 2.5 mm screen (Fig. 5.4). Although the 2.5 mm screen had some worm tangling in the screen mesh and trace amounts of soil passing through the screen (Fig. 5.4), it was rated the best of the three.

Figure 5.4. *From left to right: 0.5 mm screen with high worm entanglement after 3 hrs harvesting; 2.5 mm screen with less entanglement than the 0.5 mm screen; 2.5 mm screen allowed trace amounts of soil to pass through; the amount of soil that fell through the 3.2 mm screen just after loading the sieve with soil.*

Harvesting Experiments:
A series of trials evaluating the prototype harvester was conducted in which the soil temperature and quantity of worms harvested over time were measured for each of the three heating pad power settings (Fig. 5.5). Trials for each power setting were replicated seven times. To measure the efficiency of each harvest, a known amount of worms was put into sterile soil (containing no worms) prior to the harvesting trial and harvested worms were measured at 45 min intervals. Replicate trials were averaged together to yield a mean harvesting efficiency.

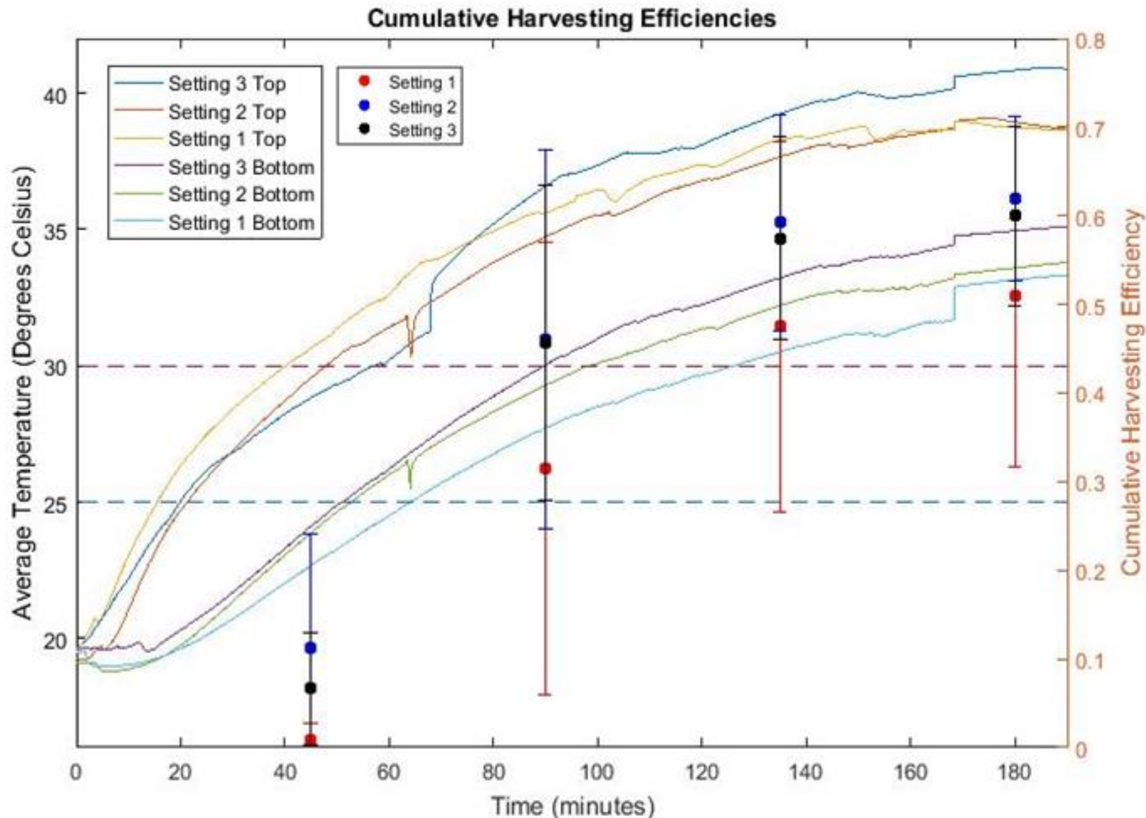


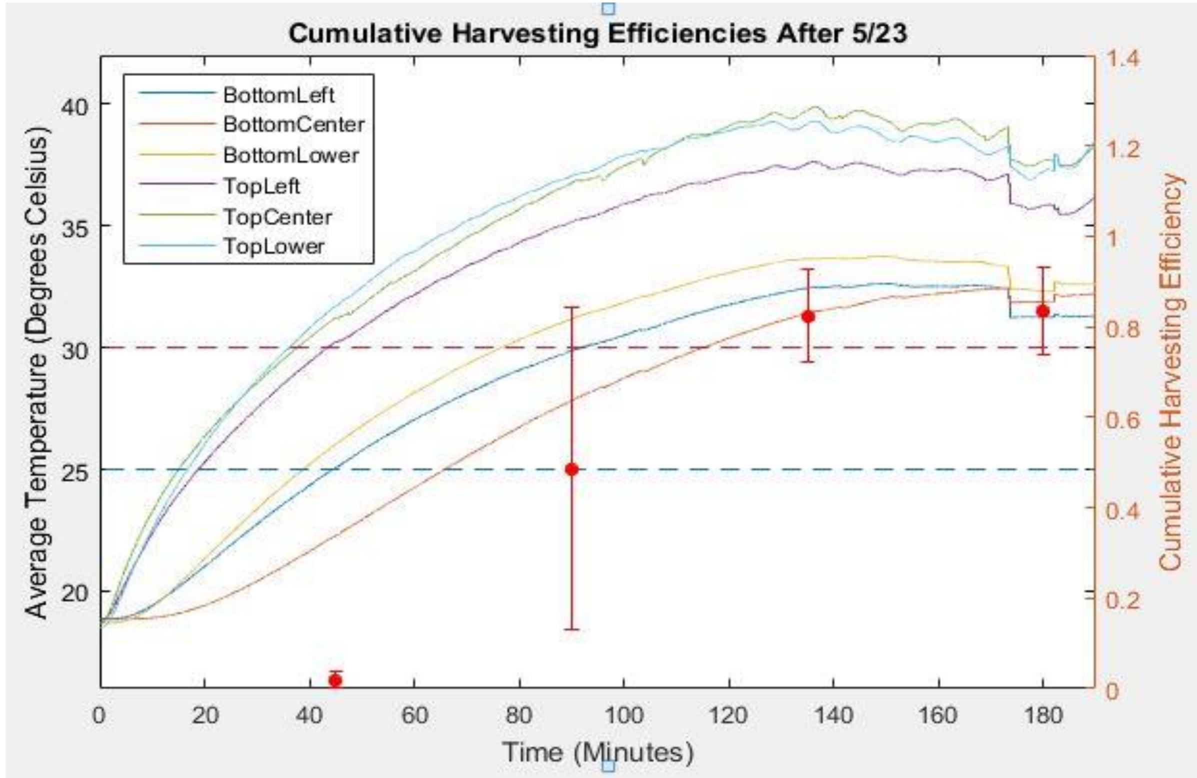
Figure 5.5. *The mean harvesting efficiency for each heating pad setting (1=low, 2=medium, 3=high) shown at 45 minute intervals along with temperature recorded every 0.5 seconds.*

Based on this first series of harvesting efficiency trials, we determined that temperature need to be controlled to maintain the soil temperatures safe for white worm eggs, as well as for automating the harvesting system. A temperature controller with a single probe, capable of being programmed to turn off at a certain temperature, was added to the prototype harvester. Two experiments were run with the probe on the bottom of the soil and set to 25 °C, and on top of the soil and set to 35 °C.

Heating the soil at the bottom of the harvester to 25 °C led to the top soil layers cooling off too quickly, which could lead to worms moving away from the heat at the bottom and migrating back to the upper layer of the soil. However, when the soil on top was heated to 35 °C, the resultant harvesting efficiency was 0.16. Therefore, in an effort to increase the harvesting

efficiency, the temperature probe setting at the soil top was increased to 40 °C. In addition, a mount for the probe was incorporated to keep it at a constant depth (5 mm) in the soil. These changes dramatically increased harvesting efficiency (Fig. 5.6).

Figure 5.6. *Mean harvesting efficiency at 45 minute intervals of six trials (red dots ± one standard*



deviation) of the prototype harvester set to 40 °C and with soil temperature recorded from top and bottom soil layers every 0.5 seconds.

To evaluate the effectiveness of the modifications made to the prototype harvester, another series of trials were conducted. At least three harvesting trials were completed for each modification treatment, with the temperature controller set to 40 °C and run for 3 hours. The modification treatments consisted of: 1) both insulation and aluminum sheet [all mods], 2) insulation only [Ins], 3) aluminum sheet only [Al], and 4) neither the aluminum sheet nor insulation [no mods]. There was no statistical significance in the harvesting efficiency between any of the modification treatments set ups (one-way ANOVA, p=0.1219) but the insulation and aluminum sheet together (all mods) yielded a 16% higher mean harvesting efficiency when compared to the set up without the aluminum sheet and insulation (no mods; Fig. 5.7).

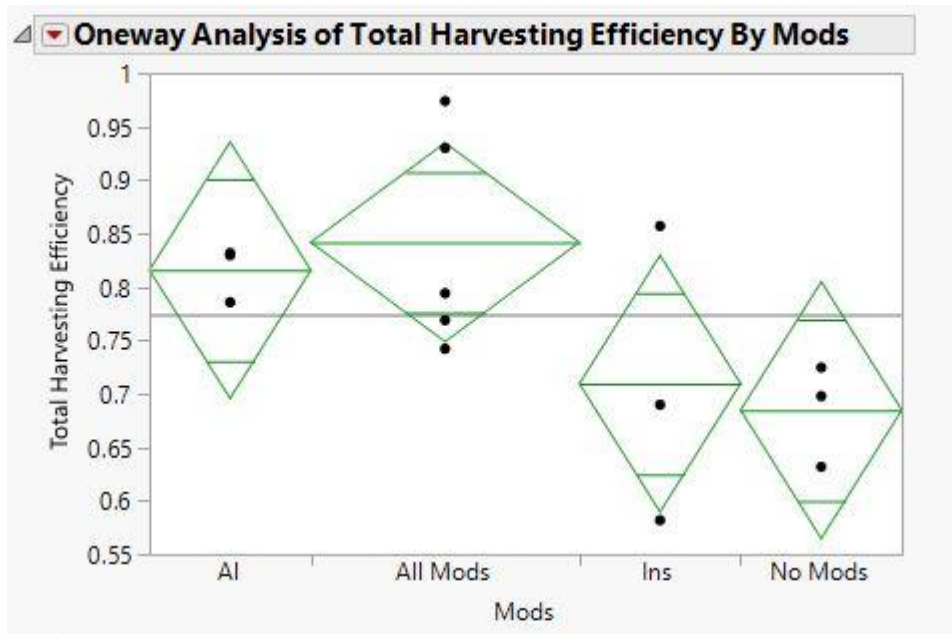


Figure 5.7. *One-way ANOVA of the mean harvesting efficiencies from each modification treatment tested. The centerline of each diamond represents the mean harvesting efficiency for each setup. The upper and lower liens in each diamond represents the standard error in the harvesting efficiency for each setup. Al = aluminum sheet only, All mods = both insulation and aluminum sheet, Ins = insulation only, No mods = neither the aluminum sheet nor insulation.*

Andrew continues to analyze the prototype worm harvesting data and make recommendations for future study. At this point, it appears of the various designs evaluated, the best overall harvesting efficiency occurs after 2.25 hrs using the prototype with aluminum and insulation modifications, 2.5 mm screen size, and surface temperature set to 40 °C.

ACCOMPLISHMENTS:

Outreach Overview
In Year 2 of the project, samples of white worms were made available to industry stakeholders to test and try in their own facilities with a variety of species. After testing the worms, the stakeholders supplied feedback by completing a survey. These surveys were analyzed to identify live white worm market(s), and from that feedback, specialized enrichment trials were conducted in Year 3 to customize the worm's nutritional profile to the predator's (namely ornamental fishes) needs. Presentations were given at Aquaculture America meetings, a paper was published in Aquaculture, and fact sheets were produced and submitted to NRAC. In addition, follow up occurred with all stakeholders who participated in the white worm research to share the project results.

Targeted Audiences
- Aquaculturists in private industry and academic institutions.
- Local food industries, especially breweries, which have waste products usable in white worm production.

Outputs:
- Based on stakeholder information from the first white worm workshop in Year 1, a list of metrics was developed for evaluating white worms as a live feed and presented as an online survey for all participants to complete after using their white worm samples.
- White worm samples were provided free during Year 2 to anyone who wanted to try feeding them to cultured or captive species. Approximately 222,530 worms were distributed to stakeholders. In addition, one starter culture (worms in media) was supplied to a grower, as well as start-up rearing instructions to several other growers.
- Contemporary methods designed to grow white worms at minimal cost were evaluated and a growing guide produced (NRAC Fact Sheet No. 223-2017).
- A prototype white worm harvester was designed, built, and tested.

Outcomes/Impacts:
Knowledge about white worms as a potential live feed amongst the aquaculture industry and scientific community has increased. A few aquaculturists have requested information about starting their own white worm cultures or have inquired to see if UNH is set up to distribute white worms.

Impacts Summary

1. **Relevance:** Issue – what was the problem?

There is a need for more diverse and nutritional live feeds. White worms show promise but not enough is known about cost-effective, scalable production techniques or nutritional profiles. In addition, the market for white worms needs to be defined.

2. **Response:** What was done?

We conducted experiments to evaluate how low- or no-cost industry byproducts affected white worm production and nutrition, and if adding enrichments would change the fatty acid profile of the worms, rendering them a more nutritious feed for cultured organisms. We evaluated if live white worms harbored any pathogens which would put aquaculture facilities at risk. We solicited feedback from stakeholders – aquaculturists in research and private domains who raise freshwater, brackish, and marine fishes – by shipping live white worm samples for them to test in their facilities. We developed live white worm shipping and receiving guidelines. We measured the conductivity properties of worm cultures (worms + media) when heated and tested other methods (besides heat) to refine and improve harvesting efficiency. We published papers, presented talks and posters, held workshops, and interacted directly with commercial ornamental growers to disseminate project results.

3. **Results:** How did your work make a difference (change in knowledge, actions, or conditions) to the target audiences?

Knowledge about white worms as a potential live feed amongst the aquaculture industry and scientific community has increased. A few aquaculturists have requested information about starting their own white worm cultures or have inquired to see if UNH is set up to distribute white worms.

4. **Recap:** One- sentence summary

White worms are an easily and cheaply cultivated, pathogen-free feed, high in protein and fat, that are readily consumed by many fishes, especially ornamentals.

PUBLICATIONS

Presentations:

Oral:

Fairchild, E. A. and J. T. Trushenski. 2018. Improving white worm *Enchytraeus albidus* nutrition for ornamental fishes. Ornamental Fish Session. The annual meeting of the World Aquaculture Society, February 19-22, 2018, Las Vegas, NV. (invited talk; accepted presentation)

Fairchild, E. A., M. Chambers, and M. L. Walsh. 2017. Do white worms have commercial potential as a feed in the ornamental industry? Ornamental Fish Session. The annual meeting of the World Aquaculture Society, February 20-22, 2017, San Antonio, TX.

Bergman, A., J. T. Trushesnki, and E. A. Fairchild. 2016. Cultivation of white worms *Enchytraeus albidus* using low- or no-cost feed resources. Aquaculture 2016. The annual meeting of the World Aquaculture Society, February 22-26, 2016, Las Vegas, NV.

Fairchild, E. A. and E. Groover. 2016. Effects of feeds and temporal cycles on white worm *Enchytraeus albidus* production. Aquaculture 2016. The annual meeting of the World Aquaculture Society, February 22-26, 2016, Las Vegas, NV.

Fairchild, E. A. 2015. Aquaculture initiatives at the Coastal Marine Lab. University of New Hampshire Department of Biological Sciences Sustainable Agriculture Seminar Series, September 18, 2015, Durham, NH.

Posters

Fairchild, E. A. and C. Giray. 2016. White worms *Enchytraeus albidus*: a pathogen-free live feed? Aquaculture 2016. The annual meeting of the World Aquaculture Society, February 22-26, 2016, Las Vegas, NV.

Peer-reviewed:

Print

Fairchild, E. A., A. M. Bergman, and J. T. Trushenski. 2017. Production and nutritional composition of white worms *Enchytraeus albidus* fed different low-cost feeds. Aquaculture 481: 16-24.

Digital

None

Non-Peer-reviewed:

Extension factsheets

Fairchild, E. A. and M. L. Walsh. 2017. How to grow white worms. NRAC Fact Sheet No. 223-2017.

Fairchild, E. A., M. L. Walsh, J. T. Trushenski, K. L. Cullen, and M. Chambers. 2017. White worms – a low cost live feed for the ornamental industry. NRAC Fact Sheet No. 224-2017.

Popular articles

World Aquaculture's editor has requested an article which we plan to write. We also worked with the science editor upon his inquiry and submitted an article for Hatchery International, however it was never published.

STUDENTS/PARTICIPANTS:

Name: Justin Roberts
Worked on project during his freshman year (Aug. – Oct. 2015).

Name: John Taylor
Worked on project during his sophomore year (Sept. 2015 – May 2016).

Name: Rachel Moore
Worked on project during her sophomore year (Sept. 2015 – May 2016).

Name: Andrew Pompeo
Whether Degree was completed during the reporting period (name, yes/no): no
New or Continuing Student: new Master's student
Capstone/Thesis Title (actual or anticipated): Design of an automated harvester to improve white worm production potential.
Date of Graduation: expected 12/2017

Name: Elizabeth Groover
Worked as summer technician May – Aug. 2016 prior to starting UFL Master's program in ornamental aquaculture.

PARTNERSHIPS

The following were unfunded partners on the project who tested a sample of live white worms in their aquaculture facilities:

- Doug Millar, TomKat Ranch, La Honda, CA
- James Candrl, Columbia Environmental Research Center, Columbia, MO
- Joe Sullivan, Tulsa, OK
- Matt DiMaggio, UFL, Ruskin, FL
- Dustin Drawdy, Oak Ridge Fish Hatchery, Plant City, FL
- Mike Bunting, Aquatic Collectors of FL, Wiamauma, FL
- Johnathon Foster, FishEye Aquaculture, Dade City, FL
- Jeff Carter, Carter's Fish Hatchery, Wiamauma, FL
- Eric Litvinoff, Marine Science Magnet High School, Groton, CT

In addition, Dr. Fiona Wilson was replaced by Dr. Tracy Keirns (UNH Survey Center) to take over the online survey tasks when Dr. Wilson assumed a new position at UNH.

When Dr. Jesse Trushenski moved from USI- Carbondale to Idaho Fish & Game, we contracted with New Jersey Feed Labs, LLC to complete white worm and feed nutritional analyses.

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 $1{\times}10^{4}$ 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.

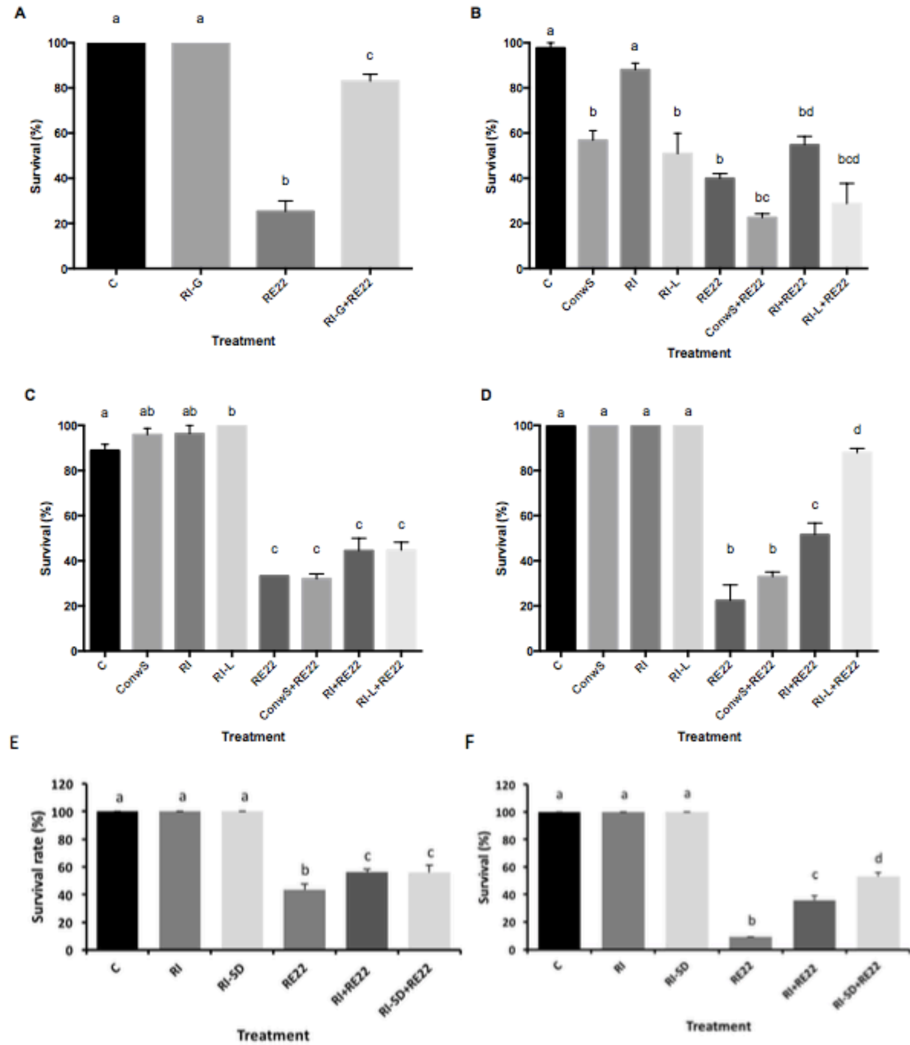


Figure 1. Laboratory challenged experiments results: Effect of pre-incubation of oyster larvae with *Bacillus pumilus* RI06-95 formulated products for 24 h on survival (% ± SEM) after challenge with *V. coralliilyticus* RE22. Survival was measured 24 h after challenge and 48 h after addition of the probiotic. (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).

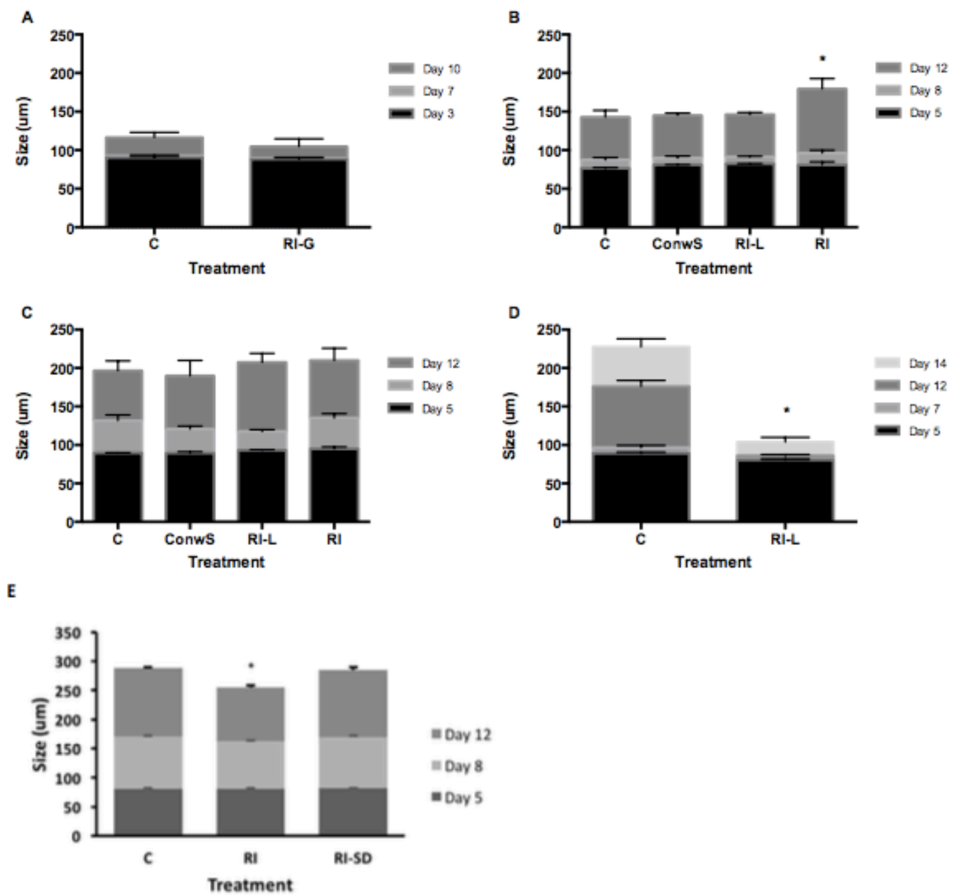


Figure 2. Effect of daily treatment with different formulations of *Bacillus pumilus* RI06-95 of larval eastern oysters (*Crassostrea virginica*) in the hatchery on mean larval size (µm ± SEM) at selected time points. (A) Trial I; (B) Trial II; (C) Trial III; (D) Trial IV and (E) Trial V. Abbreviations: 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.

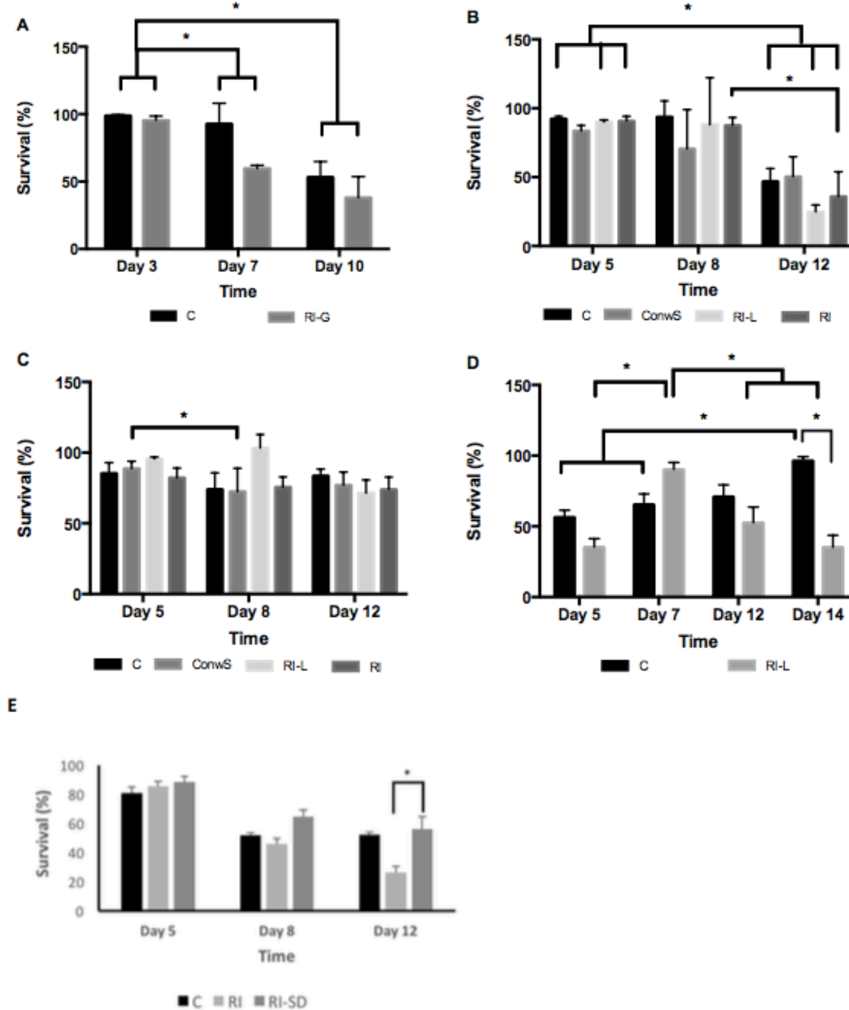


Figure 3. Effect of daily treatment with probiotics in the hatchery on interval survival (% ± SEM) of oyster larvae between selected time points. (A) Trial I; (B) Trial II; (C) Trial III; (D) Trial IV and (E) Trial V. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated formulation; 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.

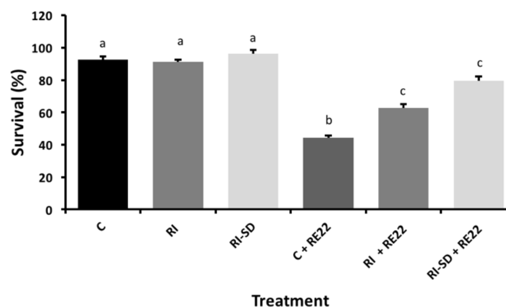


Figure 4. Effect of daily probiotic treatment in the hatchery on larval survival to a laboratory challenge with the pathogen *Vibrio coralliilyticus* RE22. Larvae were

	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

	• Date of Graduation: PhD, May 2018
Students/Participant s (URI)	• 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)
Students/Participant s (RWU)	• 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
Students/Participant s (RWU)	• Name: Nathan Canfield • Whether Degree was completed during the reporting period (name, yes/no): BS, no • New or Continuing Student: Continuing
Students/Participant s (RWU)	• 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
Students/Participant s (RWU)	• 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
Students/Participant s (RWU)	• 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
Partnerships	List any partners that you worked with on your project. Provide the following information for each Partner:

Partner	Specific Type	Level	Nature of Partnership
	Type	Level	

APPENDIX B

NRAC ANNUAL PROGRESS REPORT

Project Title	A Novel Approach to Prevent Super Chill in Atlantic salmon
Reporting Period	9/01/16 through 8/31/17
Author (Chair)	Name of person submitting this report. Deborah Bouchard for Ian Bricknell
Key Word	Atlantic salmon, super chill, osmopotentiator, simple sugars, sugar alcohols, freezing point depression
Funding Level	Total funds allocated for this project to date. *NOTE: This could be reported by Year. i.e.,* *Year One: FY 2012, $ amount* $86,451 *Year Two: FY 2013, $ amount*
Participants	*PD Ian Bricknell, PhD; Professor of Marine Science, School of Marine Science, University of Maine, 5735 Hitchner Hall, Orono, ME 04469 Business Phone/Fax: (207) 581-4380/4430 e-mail: Ian.bricknell@umit.maine.edu *Co-PI Deborah A. Bouchard, University of Maine Animal Health Laboratory Manager, Director ARI University of Maine, Cooperative Extension, 5735 Hitchner Hall, Orono, ME 04469 Business Phone/Fax: (207)581-2767/4430 e-mail: dbouchard@umext.maine.edu *Chris Bartlett, Marine Extension Associate, Marine Technology Center, City of Eastport, 16 Deep Cove Road, Eastport, ME 04631 Tel 207.853.2518 e-mail: cbartlett@maine.edu *Gary Burr PhD, Post-doctoral Research Scientist, National Cold Water Marine Aquaculture Center, National Cold Water Marine Aquaculture Center 25 Salmon Farm Road Franklin, Me, 04634 Business Phone/Fax: (207)422-2713/2723 e-mail: gary.burr@ars.usda.gov *Chong M. Lee, PhD, Professor Emeritus and Research, Department of Nutrition and Food Sciences, Food Science and Nutrition Research Center, University of Rhode Island, Kingston, RI Business Phone/Fax (401) 874-2862/2994; e-mail: chonglee@mail.uri.edu Leighanne Hawkins DVM, Cooke Aquaculture Maine P.O. Box 991 Calais , Maine 04619 Tel: (506) 755-1340 email: leighanne.hawkins@cookeaqua.com
Project Objectives	**Objective 1:** Investigate the concentrations of simple sugars and sugar alcohols (SSASA) in plasma of Atlantic salmon to achieve a higher freezing point depression limit. **Objective 2**: Investigate the uptake of simple sugars and sugar alcohols from the diet in both epithelia cells and the plasma of Atlantic salmon. **Objective 3:** Determine the efficacy of simple sugars and sugar alcohols in reducing the impact of a super chill event in Atlantic salmon under controlled conditions. **Objective 4:** Measure the physiological parameters of the fish subjected to Objective 3 and compare to control. **Objective 5:** Liaise with Atlantic salmon aquaculture industry, extension and technology transfer

Anticipated Benefits	This project will benefit the salmon aquaculture industry directly and will deliver critical research exploring a novel approach to mitigating super chill in cultured Atlantic salmon. The Northeastern US (Maine) salmon aquaculture industry has stated that super chill risk is the major limiting factor for growth of the industry. Successful completion of this project would provide the groundwork to not only mitigate current losses resulting from super chill, but it would also provide the industry with the opportunity for considerable expansion. The Maine salmon industry has stated that if super chill risk was eliminated, production potential could reach three times the current levels in Maine.
Project Progress	This update report covers the first year of the project from late September 2016 through August 2017. The project remains on target with completion of the objective's projected timeline. In particular Year 1 addressed Objectives 1, 2 and 5. **Objective 1**: Investigate the concentrations of simple sugars and sugar alcohols (SSASA) in plasma of Atlantic salmon to achieve a higher freezing point depression (FPD) limit. *Hypothesis: The addition of SSASA to the plasma of Atlantic salmon will elevate the freezing point depression in vitro.* A fundamental *in-vitro* experiment was designed to determine the levels of SSASA in plasma required to depress the FPD point below -0.7ºC which is the point at which Atlantic salmon(ATS) experience physiological collapse and mortality. To achieve this, blood from adult, seawater adapted ATS was collected. Cooke Aquaculture assisted UMaine researchers by providing transport and access to ATS being harvested. The blood was collected into heparinized tubes and centrifuged at 1,500xg and the plasma collected. The plasma was pooled and frozen at -20ºC until required. Table 1 lists the SSASA that were tested. SSASA are considered to be a supplement by the USDA as GRAS (generally recognized as safe) and are not considered a 'treatment' but part of the diet formulation. Each SSASA was added at concentrations of 0.1,1,10 and 100mM and the FPD of the SSASA enhanced plasma was determined. The SSASA plasma mixture was subjected to a FPD determination using a 6002 Touch Micro OSMETTE (PSI Precision Systems Inc.) and osmolality and the freezing point determined. This did provide the basal levels required of each SSASA tested *in-vitro* to decrease the FPD value of the plasma compared against the FPD value established for the normal plasma collected from the fish.

SSorSA*	Molecular weight	Formula
Xylose	150.13	$C_5H_{10}O_5$
Sorbitol	182.17	$C_6H_{14}O_6$
Trehalose	342.30	$C_{12}H_{22}O_{11}$
Xylitol	152.15	$C_5H_{10}O_5$
Inositol	180.16	$C_6H_{12}O_6$
Sucrose	342.30	$C_{12}H_{22}O_{11}$

Table 1 *Simple sugars and sugar alcohols that will be initially evaluated in Objective 1

Results from this experiment indicated that that all of the listed SSASA added to the ATS plasma were able lower the FPD to below -0.7ºC. All SSASA lowered the freezing point below

-0.7°C when levels reached 40mM and greater with no significant difference between any of the SSASA. It was expected that SSASA added to plasma would decrease the FPD but it was essential and beneficial to determine at what concentration and also to determine if there were any differences with particular SSASA. The results of this objective achieved these goals.

Objective 2: Investigate the uptake of simple sugars and sugar alcohols from the diet in both epithelia cells and the plasma of Atlantic salmon. *Hypothesis: Both epithelial cells and plasma will uptake SSASA from the diet of Atlantic salmon.*

Objective 2 is to assess uptake of the SSASA added to feed formulations and its duration *in vivo*. It was decided by the research team that a preliminary pilot experiment would be performed. As determined in objective 1, *in-vitro* all SSASA added to the plasma were able to lower the FPD relatively equally. For this pilot trial two SSASA were selected; trehalose (large molecular weight) and sorbitol (low molecular weight). A standardized formulation of salmon diet was made as the base diet. Four test diets were made using the base diet that added either trehalose or sorbitol at two concentrations, 2% and 10%. The aquaria based trial was carried out at the USDA's National Cold Water Marine Aquaculture Center (NCWMAC), Franklin Maine to examine the uptake of the SSASA from the diet in seawater adapted Atlantic salmon in January through April 2017. A 15-week feeding trial was conducted using the two SSASA diets and the control diet and the trial was also run with fish at two temperatures 14 ± 1 °C and 4 ± 1 °C. Fish sampling occurred at 0, 3,6, 9, 12 weeks post feeding. Feeding was stopped at week 12 and a final sampling was done at week 15 to determine duration. Again, this was a preliminary pilot study. Samples of blood and skin were taken at each time point to determine the FPD, and update and duration of trehalose and sorbitol in plasma and skin cells. Assays for determining the levels of tehalose and sorbitol in collected samples have been optimized using Megazyme's© highly sensitive and specific enzyme colorimetric assay kits. The data obtained from this study is currently in the process of being analyzed. A second larger uptake study using 4 SSASA is anticipated to begin in late October 2017 and it will also incorporate **Objective 3:** Determine the efficacy of simple sugars and sugar alcohols in reducing the impact of a super chill event in Atlantic salmon under controlled conditions.

Objective 5: Liaise with Atlantic salmon aquaculture industry, extension and technology transfer

The proposed project involves direct participation of the salmon aquaculture industry. Cooke Aquaculture's lead veterinarian, Leighanne Hawkins and US marine production manager, David Morang are current with all research progress to date. The project's first year was primarily groundwork that involved *in-vitro* testing, formulating the ATS feed and a preliminary uptake study. However, Cooke Aquaculture's production managers and lead research and development personnel traveled to the USDA/ARS to discuss the project's research progress in May and July of 2017. A formal workshop is planned at the end of the Year 2 of the project along with technology transfer.

Accomplishments:

Outreach Overview	The project's first year was primarily groundwork that involved in-vitro testing and formulating the feed. However, Cooke Aquaculture's production managers and lead research and development personnel traveled to the USDA/ARS to

<table>
<tr><td></td><td>discuss the project's research progress in May and July of 2017. A formal workshop is planned at the end of the Year 2 of the project.</td></tr>
<tr><td>Targeted Audiences</td><td>The target audience for this research is the Atlantic salmon aquaculture industry in Maine and aquaculture feed production companies. This project has direct involvement with Cooke Aquaculture, Maine major salmon aquaculture producer and a company that is a global leader in feed production. Both entities have been proactive in following the projects research results to date.</td></tr>
<tr><td>Outputs:</td><td>In Year 1 of the project, the simple sugar and sugar alcohol osmopotentiators for the diet formulations were identified. Diets for mitigating super chill in Atlantic salmon have been formulated.</td></tr>
<tr><td>Outcomes/Impacts:</td><td>Completion of Year 1, knowledge on SSASA osmopotentiators for ATS diet formulation were identified and diets have been formulated. Communication between researchers and the ATS industry has increased.</td></tr>
<tr><td>Impacts Summary</td><td>
1. Relevance: Issue – what was the problem?
Super chill is a physiological collapse in salmon occurring during periods of extreme cold weather in the Northeastern US. The Maine salmon industry has stated that super chill is a limiting factor for growth of the industry in Maine
2. Response: What was done?
To date, the simple sugar and sugar alcohol (SSASA) osmopotentiators for the diet formulations were identified. Diets for mitigating super chill in Atlantic salmon have been formulated.
3. Results: How did your work make a difference (change in knowledge, actions, or conditions) to the target audiences?
Year 2 of the project will evaluate the efficacy of SSASA in reducing the impact of super chill in a control environment.
4. Recap: One- sentence summary
The primary product/outcome is the development of diet formulation with simple sugars and sugar alcohols to reduce the impacts of super chill in cultured Atlantic salmon and allow for expansion of the ATS salmon aquaculture industry in the northeastern US.
</td></tr>
<tr><td>Publications</td><td>No formal presentations or publications to date. This is in process for Year 2</td></tr>
<tr><td>Students/Participants:</td><td>Emily Tarr; BS in Marine Sciences, Yr 3 undergraduate Student Served as a 2017 summer laboratory intern for the project. Anticipated degree date May 2019
Laurel Anderson; BS in Biology, Yr 3 undergraduate Student Served as a summer 2017 laboratory intern for the project. Anticipated degree date May 2019</td></tr>
<tr><td>Partnerships</td><td>
<table>
<tr><th>Partner</th><th>Specific Type</th><th>Level</th><th>Nature of Partnership</th></tr>
<tr><td>Cooke Aquaculture</td><td>Atlantic salmon aquaculture industry</td><td>Non-funded</td><td>Participates in all findings and results
Participates in all findings and results</td></tr>
</table>
</td></tr>
</table>

ANNUAL PROGRESS REPORT

Project Title	Testing and Application of Novel Probiotic Bacteria for Use in Marine Aquaculture
Reporting Period	9/01/2016 - 8/31/2017
Author (Chair)	Name of person submitting this report. Dennis McIntosh
Key Word	probiotic, Fundulus, bacteria, disease, finfish
Funding Level	Total funds allocated for this project to date. *NOTE: This could be reported by Year. i.e.,* *Year One: FY 2012, $ amount* $97,716 *Year Two: FY 2013, $ amount* $98,708
Participants	Name(s)/Role(s): Harold J. Schreier*, Co-PI; Eric J. Schott*, Co-PI Institution/Agency/Business: UMCES; IMET Address(s): 701 E. Pratt St., Baltimore, MD 21202 Phone(s): (410) 234-8874; (410) 234-8881 Email(s): schreier@umbc.edu; schott@umces.edu Funded (Yes/No): yes; yes
Project Objectives	This project was designed with six objectives: 1. Test the effect of probiotic candidates on larval performance of four (two freshwater and two marine) commercially important aquaculture species. 2. Measure the protective effect of probiotic candidates in the presence of pathogens. 3. Select strains having significant probiotic activity and introduce a molecular tag to aid in identification and develop a quantitative assay to facilitate year two objectives. 4. Host special session on probiotics in aquaculture at Aquaculture 2016 in Las Vegas, NV. 5. Determine dosing conditions (concentrations, dosing intervals, routes of administration) and track probiotic in intestine and culture water over time. 6. Characterize novel probiotics with respect to inhibition of pathogen growth and biofilm formation/retention.
Anticipated Benefits	One strategy for controlling disease has been to utilize probiotics, which are live microbial supplements that beneficially affect the host by modifying the host-associated microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response to disease, or by improving water quality of its ambient environment. The probiotic acts by either competing with other bacteria for essential resources or nutrients, antagonism, or by producing their own broad-spectrum antibiotics. The application appears to be useful in a wide range of life-history stages, from larvae to adults. In the aquaculture industry, the application of probiotics is not systematically used and little is known about the specific mechanisms used by individual probiotic bacteria for protection. To optimize their effectiveness, probiotics should be selected from (adapted to) the

	environment in which they will be eventually used. Fundulus heteroclitus lives in habitats with a wide range of salinities, and can be cultivated in a similarly wide range in aquaculture. Therefore, the potential probiotics that we derived from this species as part of our 2012 NRAC Mini-grant have the potential to be applied to commercially important species from a range of salinities. Impacts from this research will be applicable throughout the Northeast region and beyond for both marine and freshwater aquaculture. This work will expand our knowledge base with respect to probiotic bacteria, and thereby allow us to directly address NRAC goal TRA-13-5 Improving shellfish and finfish health maintenance and disease control.
Project Progress	**DSU** 1. Probiotic Trial 1 1. Larval Nile Tilapia were chosen for the first growth and development trials given their importance and equity in the Northeast. 2. Trial parameters included daily water quality (ammonia, nitrites, nitrates, temperature), weekly weights and mortality count. 400 tilapia (1-2 days post hatch) were fed Zeigler "starter diet" three times daily. Culture was done in 16 separate 1-L containers with a 50% daily water exchange. Each container was assigned and dosed with one of the three probiotics (two *Bacillus* spp. and one *Shewanella* spp.) previously used in trials carried out at IMET. Dosing was done at 1 microliter to maintain 10^6 concentration in culture units. Trial was terminated after 30 days due to high mortality throughout all tanks, most likely due to poor water quality due to a static system. 3. Statistical analysis showed that there was no statistically significant differences in the weights of the probiotic dosed animals versus the controls, or among the probiotic treatment groups. This has several possible reasons, one of which being tilapia's resistance to stressors (environmental as well as bacterial). Probiotic Trials 2-4 1. Three more species (clown fish, hybrid striped bass, rainbow trout) will be obtained shortly to continue the work. These three fish represent other NE species of commercial significance. 2. Larval fish have been difficult to obtain due to their cost and difficulty in rearing. The bottle neck in the species chosen is the larval stage so their worth is highest to breeders and researchers alike. Another difficult part of obtaining larval fish is the number of commercially available species that are still wild caught, while the trend is gearing toward captive bred fish and the need for probiotic treatment is high in this sector, receiving larval fish from hatcheries has been difficult. 3. Rainbow trout are laying eggs in November, eggs will be gotten the third week of November and larval fish will be in Delaware the first week of December 2017 to start the trial. Clown fish will be bred on campus, starting by the first week of December 2017. Clownfish trial will start in January 2018. Hybrid striped bass will not be breeding until the first week of March, as such eggs will be laid in March, and larval fish will be available the last week of

	March. Trials will begin during the second or third day post hatch. Trials will be completed by May 2018. 4. Three remaining trials will be carried out similarly to tilapia trial. Clown fish will be cultured in 30 ppt salinity, hybrid striped bass and rainbow trout will be 0 ppt. Water temperatures will be analogous to each fish's culture preferences and water exchanges will occur at 75%. **IMET** Cobia eggs and larvae continued to be elusive. Both IMET and DSU staff pursued contacts with Cobia hatcheries, but these did not result in any MOUs or transfer agreements. In the absence of Cobia, we used an existing relationship to obtain juveniles of European sea bass from colleagues at IMET. Trial one: Based on the lack of pathogenicity seen in the 2016 trials with Tilapia juveniles, two different *Vibrio* pathogens were used. *V. anguillarium* (M93) and *V. harveyi* (DN01). A total of 18 fish were exposed to each pathogen in 6 groups of 3. Controls were cultured similarly without any exposure to pathogen. Pathogen exposures started with a 30 minute immersion in 10^8 bacteria / ml, then the fish and bacteria (200 ml) were diluted into 2 liter vessels. Water was then exchanged (90%) 16 hours later. Fish were cultured in 16 ppt salinity at 26˚C with daily water exchanges for another 9 days. There was no mortality in controls and in *V. anguillarium*, and an insignificant mortality (=2) with *V. harveyi*. Trial two: In this trial, we used freshly hatched hybrid striped bass (Morone saxatilis x chrysops). These were procured from Delmarva Aquatics by McIntosh and Myer at DSU. After 3 days equilibration, ~1000 larvae were transported to IMET, where they were cultured at 24 ˚C and brought from 0 to 8 ppt over 4 days. Fish were fed artemia 2x/ day. A pilot experiment was conducted to test the effects of the two pathogens, *V. anguillarium* strains NB10 and M93, as well as the two probiotics, Iso11 and Iso12. Actively growing cultures of each strain were added to 20 larvae in 1-L vessels and mortality monitored for 7 days. Water quality was maintained with water exchanges, using a 53 micron filter to retain larvae in the aquarium. No mortality was observed in controls or probiotics treatments. No mortality was observed in the M93 treatment. In the NB10 treatment, larvae numbers declined from 20 to 7 by day 4, and declined to 4 by day 6. This was a significant advance, in finding a pathogenic strain and in obtaining the fish developmental stage that was most relevant to the study. A second trial was designed, using strain NB10, with and without the two probiotics. A second shipment of HSB larvae was obtained by DSU and 2000 were transported to IMET as before, but as 2 day larvae instead of 3 day larvae. These larvae suffered over 90% mortality in the equilibration to 8 ppt salinity. The reason for this was not clear, but we note that the culture density (2000 larvae in 10 gallons) was higher than the first time, and the salinity adjustment started one day earlier than before. The high mortality of these larvae made the experiment impossible to conduct. Regrettably, this was the last batch of HSB available from any supplier

	for 2017. The process is seasonal, dependent upon capture of wild reproductively active *M. saxatilis*/chrysops. While we have been pursuing larvae to carry out probiotic/pathogen challenge experiments we have been examining the molecular genetic basis for the inhibitory activities of our probiotic isolates. Although not a primary objective of our funded studies, we believe that these studies will be helpful in understanding probiosis and may lead to the generation of probiotic strains having elevated activities. Using transposon mutagenesis, we attempted to identify genes involved in probiotic activity by isolating ISO11 mutants that lost the ability to inhibit growth of Vibrio spp. strains DN01 or NB10. We successfully used this approach to identify a genetic cluster in a related probiotic strain, *B. subtilis* T1, that encoded antimicrobial polyketides and lipopeptides. Unfortunately, ISO11 carries multiple drug resistance determinants, which interfere with our ability to select for mutants by traditional methods. The presence of chloramphenicol, erythromycin and spectinomycin resistance determinants were confirmed in the ISO11 genome, which we have recently sequenced. The genome sequence provides us with a tool to explore the mechanisms associated with ISO11 probiotic activity as part of our future studies. Since possession of a model system that could be used to analyze probiotic activity at any time of the year would be beneficial, we have been studying the use of *Artemia fansciscana* nauplii for our probiotic efficacy studies. The use of Artemia to analyze probiotic/pathogen interactions has been documented and cultures of these brine shrimp are available daily from the IMET Aquaculture Research Center. We have been developing procedures to examine the effect of ISO5, ISO11, and ISO12 on Artemia growth and protection against pathogen strain NB10. Unfortunately, our control treatments (unchallenged Artemia) have exhibited high mortality, which we have attributed to the source of Artemia food used in these experiments. This problem will be addressed using published protocols, including via the feeding of cultures of *Aeromonas hydrophila*, which we recently obtained from the University of Ghent.
Accomplishments:	
Outreach Overview	Efforts to share this knowledge include formal presentations at Aquaculture America 2017, the 2017 ARD Research Symposium by DSU Graduate Students Jasmine Smalls and Jackie Myer (see below). In addition, this project is regularly shared with tour groups visiting the DSU Aquaculture Research and Demonstration Facility. Visitors include K-12 teachers and students from across Delaware, various local and state policy makers, members of the research community, as well as current and prospective aquaculture producers.
Targeted Audiences	Direct end users of the knowledge generated by this project will be members of the research community that are exploring the potential of probiotics for use in aquaculture to enhance production, improve nutrient use efficiency and/or mitigate disease impacts.
Outputs:	Graduate student Jackie Myer was sent to Virginia Tech Agricultural Research and Extension Center (June 2017) to participate in a larval rearing class. Class centered mostly around clown fish and live feed techniques required for many larval fish, but included other species

	including cobia and rainbow trout. Class was taught by VT's lab manager, Steve Urick, and was hands on in their lab.
Outcomes/Impacts:	Following our presentation at Aquaculture 2016 in Las Vegas in February, we were approached by a representative from Epicore Bionetworks, Inc. (http://www.epicorebionetworks.com/). They are interested in licensing our probiotics for potential commercialization. Schreier and Schott are currently collaborating with Epicore on the effects of probiotics on shrimp challenged with Acute Hepatopancreatic Necrosis Disease (*V. parahaemolyticus*).
Impacts Summary	1. **Relevance:** Issue – what was the problem? One strategy for controlling disease has been to utilize probiotics which are live microbial supplements that beneficially affect the host. The probiotic acts by either competing with other bacteria for essential resources or nutrients, antagonism, or by producing their own broad-spectrum antibiotics. While the application appears to be useful in a wide range of life-history stages, from larvae to adults, the application of probiotics is not systematically used in the aquaculture industry. 2. **Response:** What was done? Growth and survival trials are underway at the DSU ARDF: having already completed our work with tilapia. Challenge trials with *Vibrio* pathogens were conducted at IMET with sea bass, tilapia and hybrid striped bass (collaborators Schott and Schreier). The project has increased the institutional capacity of IMET to conduct vertebrate pathogen challenges. 3. **Results:** How did your work make a difference **(change in knowledge, actions, or conditions)** to the target audiences? Our work is not yet at a point where the technologies being developed could be put into practical use at the farm level, though we have begun discussions with multiple companies interested in commercializing our results. The interest that we have 4. **Recap:** One- sentence summary Applying preventive measures may lead to less reliance on the use of chemicals - disinfectants, pesticides and antimicrobials - that treat the symptoms of the problem rather than affecting a cure.
Publications:	• Oral Presentations: o Smalls, J. and D. McIntosh. The Use of Probiotics in Shrimp Aquaculture. Aquaculture America 2017 Book of Abstracts, San Antonio, TX, USA. o Myer, J. L., and D. McIntosh. Probiotics and Fish Growth. 2017 ARD Research Symposium, Atlanta, GA. o Smalls, J. and D. McIntosh. The Use of Probiotics in Shrimp Aquaculture. 2017 ARD Research Symposium, Atlanta, GA.

- Poster Presentations
  - Myer, J. L., E. Schott, H. J. Schreier, and D. McIntosh. Probiotics and Fish Growth. Aquaculture America 2017 Book of Abstracts, San Antonio, TX, USA
- Peer-reviewed:
  - Print (journal, etc.) - none to report at this time
  - Digital (websites, videos, etc.) - none to report at this time
- Non-Peer-reviewed:
  - Extension factsheets - none to report at this time
  - Popular articles - none to report at this time

Students/Participants:

Delaware State University

Name	**Level**	**Completed**	**New/Continuing**	**Thesis**	**Graduation**
Jasmine Smalls	MS	No	Continuing	The Use of Probiotics in Pacific White Shrimp (*Littopenaeus vannamei*) Aquaculture	May 2018
Jackie Myer	MS	No	Continuing	The effect of probiotics in four commercially important finfish in the Northeast	May 2018
Charles Wallace	BS	No	Continuing	NA	May 2018
Diondre Oliver	BS	No	New	NA	May 2018
Dean Johnson	BS	No	Continuing	NA	NA

UMD Summer Interns

Name	**Level**	**Completed**	**New/Continuing**	**Thesis**	**Graduation**
Sarah Avery	MS	No	New		NA
Susannah Ruzbarsky	BS	No	Continuing	NA	NA
Amanda Hise	BS	No	New	NA	December 2018

Partnerships:

Tilapia (Steve Hughes, Cheyney University)
Tilapia (Jeff Reeser, Baltimore Polytechnic High School)
Rainbow Trout (Steve Hughes, Cheyney University)
Clownfish (Mid-Atlantic Aquatic Technology)
Hybrid Striped Bass - (Skip Bason, Delmarva Aquatics)
Sea bass (Keiko Saito, IMET)

ANNUAL PROGRESS REPORT

Project Title	Development and evaluation of novel, non-toxic solutions for biofouling control and predator exclusion in shellfish aquaculture
Reporting Period	9/01/2016- 8/31/2017
Author (Chair)	Sandra E. Shumway
Key Word	Aquaculture, biofouling, prevention, shellfish
Funding Level	Total funds allocated for this project to date. *NOTE: This could be reported by Year. i.e.,* *Year One: FY 2016, $ amount* $91,268 *Year Two: FY 2017, $ amount* $102,314
Participants	**Name(s)/Role(s):** Sandra E. Shumway/PI Institution/Agency/Business: University of Connecticut Address(s): Dept. Marine Sciences, 1080 Shennecossett Road, Groton, CT 06340 Phone(s): 860-405-9282 Email(s): Sandra.shumway@uconn.edu Funded (Yes/No): yes **Name(s)/Role(s):** Stephan Bullard/PI Institution/Agency/Business: University of Hartford Address(s): University of Hartford, 200 Bloomfield Ave, West Hartford, CT 06117 Phone(s): 860-803-6423 Email(s): Bullard@hartford.edu Funded (Yes/No): yes **Name(s)/Role(s):** Tessa Getchis/PI Institution/Agency/Business: Connecticut SeaGrant Address(s): Connecticut SeaGrant, 1080 Shennecossett Road, Groton, CT 06340 Phone(s): 860-405-9104 Email(s): tessa.getchis@uconn.edu Funded (Yes/No): yes **Name(s)/Role(s):** Alex Walsh/Subcontractor Institution/Agency/Business: ePAINT CO. Address(s): 25 Research Road, East Falmouth, MA 02536 Phone(s): 800-258-5998 Email(s): alex@epaint.net Funded (Yes/No): yes
Project Objectives	1) Develop and refine new coatings to prevent the development of biofouling and predation on aquaculture gear; 2) Assess the potential toxicity of the new coatings. The base materials being tested have previously been shown to be non-toxic and are all cleared with FDA and EPA regulations; however, it is important to test any new configurations to confirm non-toxic status; 3) Assess the adhesion of the newly developed formulations on test panels and gear;

	4) Assess the efficacy of newly developed coatings on test panels and gear at 4 locations (ME, MA, CT, and NH) and other farms as possibilities permit (see letters of support); 5) Assess the ability of coatings to deter predation; 6) Engage aquaculture producers in research and outreach, and disseminate the results to the industrial and scientific communities through presentations at workshops, conferences, outreach publications, web page, and peer-reviewed publications.
Anticipated Benefits	Biofouling is one of the most labor intensive aspects of shellfish aquaculture, and a significant amount of time and economic resources are devoted to removing biofouling from both gear and the cultured shellfish. Biofouling significantly impacts water flow, and therefore, biofouling requires constant attention, especially during warmer months and with gear closer to the top of the water column. The majority of the time, removing biofouling is the primary reason for handling the gear, and therefore results in increased expenses, and therefore reduced profitability. Shellfish farmers often are looking for innovative ways to deal with the issue of biofouling; however, the methods currently available are either toxic to the environmentally sensitive filter feeders, or require large amounts of labor. A new method which does not require periodic dips, environmentally toxic substances or manual cleaning would significantly increase productivity and reduce the amount of time spent tending gear. This will result in higher profit per unit effort, which will result in greater investment, greater expansion of the industry, and a greater chance at economic viability of shellfish farms throughout the Northeast.
Project Progress	<u>OBJ. 1, 3 and 4 Develop and refine new coatings and assess the adhesion of the newly developed formulations on test panels and gear; Assess efficacy of newly developed coatings on test panels and gear</u> Different formulations of antifouling coatings were tested on aquaculture facilities in Massachusetts and Florida and on the docks at the University of Connecticut. All panels were deployed in triplicate. A photographic summary is provided for the panels from 2016 (after 4 months of exposure) as these were still in the water and not available for the previous report. A set of photographs for experimental deployments to date is also provided for the 2017 season. These include panels deployed in May of 2017 and a second set with newly developed coatings deployed in August of 2017. This second set is still in the water and will be summarized in the final report. **2016 NRAC** **PANEL TESTING** **NRAC Y2** Antifouling properties of biologically active polymers developed in the

first year of research were evaluated. Bio-based polymers were applied to PVC panels that were attached to PVC racks and deployed at three test sites:, 1) Senator George Kirpatrick Marine Lab (Cedar Key, FL), 2) WARD Aquafarms (North Falmouth, MA), and 3) University of Connecticut Avery Point (Groton, CT). Three replicates were tested at each site for each test coating.

Bio-based coatings were formulated with chitosan-based polymers blended with extender pigments such as calcium carbonate, magnesium silicate, zinc oxide, and combinations thereof. This novel combination of materials was used as the base formula. Phytochemicals, disclosed in the previous progress report, were added to this base formula to determine the effect on biofouling resistance. The commercially available algaecide, zinc pyrithione (CAS# 13463-41-7) was also tested to determine the relative activity of phytochemicals to a known biocide. Bioactive compounds were added to the base formula at 5% by weight. A photoactive coating marketed under the tradename NETMINDER® by NETMINDER LLC (netminder.us) as a non-toxic biofouling release coating was tested. A commercially available copper-based (25%) antifouling paint (flexdel.com) marketed to the fish farming industry for use on fish pen nets was also tested. Untreated PVC panels were used as controls.

NRAC PANELS

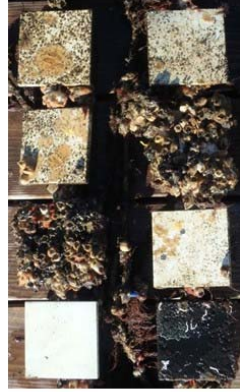
SITE	RACK	TEST COATING	PANEL
CEDAR KEY	FN-1	Bio-based resin	1100
CEDAR KEY	FN-1	Zinc oxide based	2053
CEDAR KEY	FN-1	25% Copper-based	2063
CEDAR KEY	FN-1	5% Capsaicin	2075
CEDAR KEY	FN-1	5% Birch oil	2089
CEDAR KEY	FN-1	5% Menthol	2097
CEDAR KEY	FN-1	5% Zinc pyrithione	2108
CEDAR KEY	FN-2	Bio-based resin	1106
CEDAR KEY	FN-2	NETMINDER	2054
CEDAR KEY	FN-2	25% Copper-based	2062
CEDAR KEY	FN-2	5% Capsaicin	2080
CEDAR KEY	FN-2	5% Birch oil	2086
CEDAR KEY	FN-2	5% Menthol	2092
CEDAR KEY	FN-2	5% Zinc pyrithione	2102
CEDAR KEY	FN-3	Bio-based resin	1102
CEDAR KEY	FN-3	NETMINDER	2049
CEDAR KEY	FN-3	25% Copper-based	2069
CEDAR KEY	FN-3	5% Capsaicin	2076
CEDAR KEY	FN-3	5% Birch oil	2082
CEDAR KEY	FN-3	5% Menthol	2096
CEDAR KEY	FN-3	5% Zinc pyrithione	2106

SITE	RACK	TEST COATING	PANEL
FALMOUTH	DN-1	Bio-based resin	1096
FALMOUTH	DN-1	NETMINDER	2056
FALMOUTH	DN-1	25% Copper-based	2060
FALMOUTH	DN-1	5% Capsaicin	2072
FALMOUTH	DN-1	5% Birch oil	2085
FALMOUTH	DN-1	5% Menthol	2091
FALMOUTH	DN-1	5% Zinc pyrithione	2107
FALMOUTH	DN-2	Bio-based resin	1103
FALMOUTH	DN-2	Zinc oxide based	2052
FALMOUTH	DN-2	25% Copper-based	2061
FALMOUTH	DN-2	5% Capsaicin	2073
FALMOUTH	DN-2	5% Birch oil	2087
FALMOUTH	DN-2	5% Menthol	2093
FALMOUTH	DN-2	5% Zinc pyrithione	2105
FALMOUTH	DN-3	Bio-based resin	1098
FALMOUTH	DN-3	Zinc oxide based	2048
FALMOUTH	DN-3	5% Capsaicin oil	2079
FALMOUTH	DN-3	5% Birch oil	2083
FALMOUTH	DN-3	5% Menthol	2098
FALMOUTH	DN-3	5% Zinc pyrithione	2104
FALMOUTH	DN-3	25% Copper-based	2060

SITE	RACK	TEST COATING	PANEL
UCONN	UJ-1	Bio-based resin	1105
UCONN	UJ-1	NETMINDER	2057
UCONN	UJ-1	25% Copper-based	2065
UCONN	UJ-1	5% Capsaicin oil	2078
UCONN	UJ-1	5% Birch oil	2088
UCONN	UJ-1	5% Menthol	2090
UCONN	UJ-1	5% Zinc pyrithione	2103
UCONN	UJ-2	Bio-based resin	1107
UCONN	UJ-2	NETMINDER	2050
UCONN	UJ-2	25% Copper-based	2070
UCONN	UJ-2	5% Capsaicin oil	2077
UCONN	UJ-2	5% Birch oil	2084
UCONN	UJ-2	5% Menthol	2095
UCONN	UJ-2	5% Zinc pyrithione	2109
UCONN	UJ-3	Bio-based resin	1104
UCONN	UJ-3	NETMINDER	2059
UCONN	UJ-3	25% Copper-based	2067
UCONN	UJ-3	5% Capsaicin oil	2074
UCONN	UJ-3	5% Birch oil	2081
UCONN	UJ-3	5% Menthol	2094
UCONN	UJ-3	5% Zinc pyrithione	2101

MA Panels

Rack DN1	
2093	2073
2087	2052
Control	2105
1103	2061



Rack DN2	
2048	2083
2098	1098*
Control	2079
2104	2068

Rack DN3	
Control	2056
2060	2072
2107	1096
2085	2091

FL PANELS

Rack FN1	
1100	2075
2063	2053
2089	2108
Control	2097

Rack FN2	
2086	1106
2092	2062
2102	2080
Control	2054

Rack FN3	
2106	Control
2096	1102
2069	2049
2082	2076

UCONN PANELS

Rack UJ1	
2090	1105
Control	2057
2103	2088
2078	2065

Rack UJ2	
2077	Control
2109	2050
2095	2084
1107	2070

Rack UJ3	
2074	2101
2067	2094
2059	1104
Control	2081

Results from Antifouling Tests

Senator George Kirpatrick Marine Lab (Cedar Key, FL)
Barnacle fouling in Florida waters is prolific. The best performing coating (panels 2018, 2012 and 2016) is zinc pyrithione based. This active ingredient deters marine growth more effectively than the copper-based antifouling paint. Capsaicin filled formulas (panels 2075, 2080, and 2076) also deterred barnacles more effectively than copper and the other phytochemicals.

WARD Aquafarms (North Falmouth, MA)
Barnacles, bryozoans (both filamentous and encrusting), and tube worms are the primary biofouling organisms observed at the WARD Aquafarm site in North Falmouth, MA. The zinc pyrithione based coating (panels 2105, 2104, and 2107) is the best performing. Copper-based antifouling paint resists biofouling better in Massachusetts waters than in Florida, however the zinc pyrithione based panels are virtually free of growth. Birch oil (panels 2087, 2083, and 2085) appears more biologically active than other phytochemicals.

UCONN Avery Pont (Groton, CT)
Percent biofouling coverage was calculated after three months exposure from the dock at the University of Connecticut, Avery Point Campus.

UCONN PANEL RESULTS

% Cover Data after 3 months

20-Sep-16

Crust = Quality of photos somewhat low, a brownish crust was visible on some, probably ascdian like Diplo, but hard to tell, also could be mostly bare; if I couldn't be sure it was bare, I called it crust

Some crust my be schizoporella or cryptosula, some cryptosula was seen on some panels

Unknown = in some cases high relief of surrounding material casts shadows making assesement of the substrata impossible

Encr Bry = Encrusting Bryozoan

Rack 1	Bare	Tunicate	Bryozaon	Hydroid	Sponge	Crust	Barnacle	Worm	Algae	Encr Bry	Unknown		% Bare	% Cover
Control	0	7	93	0	0	0	0	0	0	0	0	100	0.0	100.0
1105	24	9	50	0	0	1	0	0	0	16	0	100	24.0	76.0
2057	18	19	10	0	0	53	0	0	0	0	0	100	18.0	82.0
2065	6	72	21	0	0	0	1	0	0	0	0	100	6.0	94.0
2078	17	32	49	0	0	2	0	0	0	0	0	100	17.0	83.0
2088	11	3	28	0	0	47	0	0	0	7	4	100	11.0	89.0
2090	5	0	9	0	0	86	0	0	0	0	0	100	5.0	95.0
2103	98	0	0	0	0	2	0	0	0	0	0	100	98.0	2.0

*2065 = appeared mostly bare, but seems to have been almost completely covered with Diplosoma (all uncertain points = ascidians)

Rack 2	Bare	Tunicate	Bryozaon	Hydroid	Sponge	Crust	Barnacle	Worm	Algae	Encr Bry	Unknown		% Bare	% Cover
Control	0	17	82	0	1	0	0	0	0	0	0	100	0.0	100.0
1107	2	14	82	0	0	2	0	0	0	0	0	100	2.0	98.0
2050	7	15	14	0	0	64	0	0	0	0	0	100	7.0	93.0
2070	15	80	4	0	0	0	0	1	0	0	0	100	15.0	85.0
2077	15	7	13	0	0	58	0	0	0	6	1	100	15.0	85.0
2084	10	9	15	0	0	65	1	0	0	0	0	100	10.0	90.0
2095	14	0	33	0	0	48	0	0	0	5	0	100	14.0	86.0
2109	100	0	0	0	0	0	0	0	0	0	0	100	100.0	0.0

*2070 = appeared mostly bare, but seems to have been almost completely covered with Diplosoma (all uncertain points = ascidians)

Rack 3	Bare	Tunicate	Bryozaon	Hydroid	Sponge	Crust	Barnacle	Worm	Algae	Encr Bry	Unknown		% Bare	% Cover
Control	0	35	64	0	0	0	0	0	0	0	1	100	0.0	100.0
1104	13	13	69	0	0	0	0	0	4	0	1	100	13.0	87.0
2059	12	14	33	0	0	41	0	0	0	0	0	100	12.0	88.0
2067	0	23	75	0	0	0	0	0	0	0	2	100	0.0	100.0
2074	43	10	19	0	0	17	0	0	0	11	0	100	43.0	57.0
2081	31	5	29	0	0	35	0	0	0	0	0	100	31.0	69.0
2094	7	1	16	0	0	71	0	0	0	5	0	100	7.0	93.0
2101	99	0	0	0	0	1	0	0	0	0	0	100	99.0	1.0

Field Testing

Promising test formulas were applied to wooden FLUPSY, PVC coated wire and HDPE bags and exposed at WARD Aquafarms in the spring of 2017. Testing is ongoing.

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LIGHT ATTENUATION MEASUREMENTS

Testing in Florida has consistently given results that differ from other areas (see Figure X). Barnacles are a particular problem, but fouling overall is heavy and rapid. The nearshore waters in the test areas are shallow, regularly disturbed, and highly turbid. Given that the test coatings are photoactive, i.e. light-activated, it was hypothesized that lack of light penetration might be a factor limiting the efficacy of the coatings. Light meters with continuous recorders were placed in submersible casings and deployed at the same sites as the test panels in Florida and at the University of Connecticut (control). While it appears that there was slightly less light penetration at the Florida site, none of the levels at either site were low enough to limit the efficacy of the coatings (Walsh, pers. comm.). We believe it is the specific composition of the fouling community in Florida that is the determining factor.

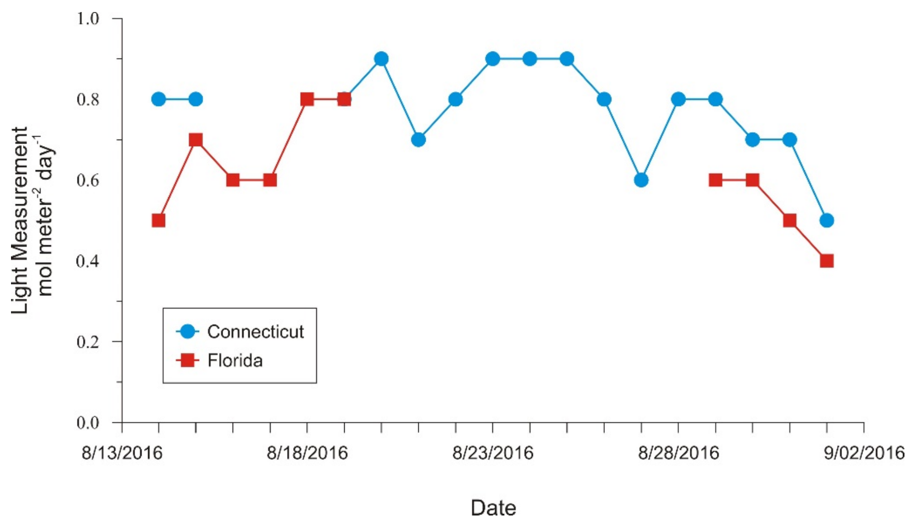

Figure 1 Light meter readings from Florida and Connecticut deployment sites.

<u>OBJ. 5: Predator Assays</u>

No further experiments were carried on this aspect of the study. While preliminary studies were promising and there were potential differences noted between the willingness of animals to cross barriers with different coatings, it became readily apparent that a proper study would require

	far more time and effort than could be supported by the present grant. We still believe that the possibility of incorporating anti-predator substances in the anti-fouling coatings is an option that should be explored further. *OBJECTIVE 6 – see below.*
Outreach Overview	See below – one presentation already given and one workshop for industry being planned for December, 2016.
Targeted Audiences	Results obtained from this research will reduce the costs and effort associated with biofouling on aquaculture farms.
Outputs:	An industry-focused WORKSHOP is being planned for the Northeast Aquaculture Conference and Exposition (NACE) in December, 2016. The Workshop is being organized by Getchis and Shumway to inform industry of current results and solicit input and recommendations based on their observations from the first season of testing. Details provided in attachment. **Industry-focused workshop** Objective 4. Engage aquaculture producers in research and outreach, and disseminate the results to the industrial and scientific communities through presentations at workshops, conferences, outreach publications, web page and web conference, and peer-reviewed publications. Overview The primary goal of the outreach effort was to work towards improved aquaculture producer knowledge on various aspects of marine biofouling. The project investigators developed a PowerPoint presentation, "Biofouling 101" and accompanying script containing science-based information on the following topics: - Identification of common marine biofouling species - Biology and ecology of common marine biofouling organisms - Effects on biofouling on various gear types and species - Available management (prevention and control) strategies - How to report new or unusual biofouling organisms The "Biofouling 101" presentation was piloted at the Northeast Aquaculture Conference & Exposition held in January 2017 in Providence, Rhode Island. The purpose of the workshop was to: (1) assess attendee knowledge on the topic, (2) provide basic information on the aforementioned topics, and finally, (3) evaluate whether or not attendee's knowledge on these topics improved following the

	presentation. The workshop commenced with distribution of a pre-test on biofouling topics. After attendees completed the pre-test, it was collected and the project investigators began the presentation. The workshop began with an overview on the five biofouling topics given by researchers and outreach staff. This was followed by presentations given by aquaculture producer which highlighted the results of field trials of biofouling coatings (the focus of this research project) on their individual farms. Workshop participants had the opportunity to view coated and uncoated gear pieces, and ask cooperative farmers about their experiences with the different antifouling coatings. Researchers were on hand to listen to suggestions with respect to product development and future directions for biofouling research and to answer questions. There was a total of 57 attendees and 7 speakers involved in this workshop. Of the total number of attendees, 30 pre- and post-tests were submitted and 24 of those surveys were considered complete. The response rate was 42%. In all cases, participants scores were higher on the post-test than on the pre-test. Workshop attendees also provided a considerable amount of input regarding their own experiences with anti-fouling strategies, and suggested future directions for anti-fouling research. In the short-term (within the project period), we are making a concerted effort to outreach the results (indicating benefits and tradeoffs of the new antifouling coating) to growers and grower associations. Additionally, the project investigators will assemble a final project report, and at least one peer-reviewed journal article that describes the research effort, results, and implications for shellfish aquaculture producers. Future work In the longer term (within the first 2-5 years following the grant period), the PI will be tracking the use and acceptance of the new coating through product sales and consumer comments. These results will be reported back to NRAC in periodic impact reports. **Accomplishments** Getchis, T.L., Shumway, S.E., Walsh, A., Bullard, S. 2017. Farmer to Farmer: What Works and Doesn't When It Comes to Biofouling Control (Workshop). Northeast Aquaculture Conference & Expo, Providence, Rhode Island.

	Anticipated Outcome(s)/Impact(s) The overall focus of this project was to develop and make available an alternative hazard management strategy (prevention as opposed to removal) for marine biofouling in aquaculture. In the short-term, we were able to expand aquaculture producer understanding about various aspects of biofouling, including promising strategies to help prevent its occurrence, and ultimately resulting in a significant cost-savings to the producer. In the longer term, adoption of this management strategy should result in a reduction in effort necessary to address biofouling; and in some cases, product will be of better quality and more valuable in the marketplace.
Outcomes/Impacts:	The following outcomes/impacts are planned, industry members are currently engaged in testing in several areas (see Project Progress) and routine interaction is integral to the project. **Short term** • An alternative hazard management strategy (prevention as opposed to removal) is developed and made available to address biofouling and predation • Producers are more aware of ways to manage biofouling and predation **Medium term** • Adoption of this management strategy (product use) results in a reduction in effort (hours spent) necessary to address biofouling and predation; in some cases, product is of better quality and more valuable in the marketplace • Producers are more easily able to identify biofouling species, when and how they can affect aquaculture operations, recognize new species and know who to notify (e.g. natural resource managers and extension professionals) **Long term:** • A significant cost savings to the producer • Biofouling is no longer considered one of the most costly problems in aquaculture
Impacts Summary	1. **Relevance:** Biofouling in shellfish aquaculture 2. **Response:** Coatings are being developed and field-tested to mitigate fouling 3. **Results:** The work is in the first season and results will not be available until the end of the fouling season in October/November 4. **Recap:** Coatings are being developed and field tested to mitigate biofouling in shellfish aquaculture.
Publications	• Presentations:

	Numerous presentations were made at formal conferences, seminars, and industry gatherings. PI Walsh, Shumway, Bullard, and Getchis participated in the Biofouling Workshop for Industry organized by Tessa Getchis at the NACE meeting in Providence, RI in December, 2016. The outcomes of that workshop are summarized below. Oral and Poster presentations were made at the US Aquaculture Meeting in San Antonio, Texas, in February, 2017, the National Shellfisheries Association Annual Conference in Knoxville, Tennessee, in March, 2017, and the International Pectinid Workshop in Portland, Maine, in April of 2017. PI Shumway had the opportunity to make presentations at many national and international venues as part of other invited activities, i.e. at no cost to the project. These included: Ocean University Shanghai, China and an aquaculture farm (May, 2016), FENAOSTRA (National Oyster Fair), an industry exposition in Florianopolis, Brazil (September 2016), a lecture as an instructor in the Erasmus Mundas Graduate Training Program in Aquaculture, University of Nantes, France (November 2016), a presentation at the City University of Hong Kong and two aquaculture farms, Hong Kong, China (May, 2017), and seminars at The Kenneth K. Chew Center for Research and Restoration, NOAA Northwest Fisheries Science Center Seattle, Washington (July, 2017), and the University of Maine at Machias (August, 2017). The NRAC grant was clearly identified as a funding source at each presentation. o
Students/ Participants:	none
Partnerships	List any partners that you worked with on your project. Provide the following information for each Partner:

Partner	Specific Type	Level	Nature of
Dan Ward Leslie Sturmer	Cooperating testing		