Project Completion Report
Project Title:

Subaward # Z51470G
Grant # 5207982

PROJECT CODE: 5404001 **SUBCONTRACT/ACCOUNT NO: Z51470G**

PROJECT TITLE: Investigations into the Potential Health and Economic Benefits of bivalve/finfish co-culture

DATES OF WORK: 6-1-08 to 12-22-10

PARTICIPANTS: Funded cooperating personnel and institutions, agencies, and business entities including extension liaison(s) and non-funded collaborators.

University of Maine
Funded
Dr Sally Molloy
Ms Deborah Bouchard
Mr. Michael Pietrak
Co-operating
Prof Ian R Bricknell

Rutgers University
Dr David Bushek
Dr Susan Ford
Ms BJ Landau (resigned from the project Fall 08)

REASON FOR TERMINATION: Indicate objective(s) completed, funds terminated, or other specific reason for project termination.

The project was terminated as the primary objectives of the proposal were completed.

PROJECT OBJECTIVES: List objectives as written in approved proposal.

Objective 1/Milestone 1: Selection and optimization of molecular procedures;
Objective 1/Milestone 2: Optimization of culture techniques and quality control program established;
Objective 2/Milestone 3: Mussel/Pathogen interaction experiments;
Objective 2/Milestone 4: Mussel/Pathogen/Fish interaction experiments;
Objective 3/Milestone 5: Seasonally placed sentinel mussels at selected finfish sites;
Objective 3/Milestone 6: Placement of sentinel mussels at farms experiencing disease outbreaks;
Objective 4/Milestone 7: Extension component.

ANTICIPATED BENEFITS: State how the project will benefit the aquaculture industry either directly or indirectly.

This project has begun a foundation of knowledge on how fish pathogens of various physiologies interact with mussels on an Integrated Multi-Trophic Farm. It is anticipated that this knowledge will help to facilitate the introduction and adoption of IMTA by finfish growers in the Northeast Region.

PRINCIPAL ACCOMPLISHMENTS: Summarize in a concise form the findings for each objective for the duration of the project. Measurement data are to be given in SI units. However, to minimize confusion, a dual system of measurement may be used to express results.
Objective 1/Milestone 1: Selection and optimization of molecular procedures;

Primers were designed to quantify expression of a constitutive house keeping or normalizing gene in tissues of the blue mussel. These primers were optimized for use in a quantitative RT-PCR (qRT-PCR) assay to be used alongside a qRT-PCR assay designed to quantify ISAV RNA.

Objective 1/Milestone 2: Optimization of culture techniques and quality control program established;
Culture techniques needed for virus cultivation and virus detection were optimized and standardization of culture techniques to be used in the evaluations. A strain of pathogenic *Vibrio anguillarum* O2β carrying a plasmid that encodes an ampicillin resistance gene and a red fluorescent protein gene was acquired from Dr. Singer's lab at the University of Maine. Culture techniques for cultivation and detection of the bacterium in mussel and cod tissues were optimized and standardized.

Standardized procedures for conducting molecular assays on split samples at both Rutgers and Maine and to ensure quality control were established. Dr. Ford and a technician were able to visit Maine and learn how to conduct the ISAV real-time RT-PCR assay and finalize the protocols for split sampling and quality control. The quality control program was in place for all of the mussel/pathogen experiments and the sentinel mussel sampling.

Objective 2/Milestone 3: Mussel/Pathogen interaction experiments;
Multiple mussel exposure trials with ISAV and *V. anguillarum* were carried out. Mussels remove *V. anguillarum* from the water column and concentrate viable bacteria in their digestive gland tissues (4.5×10^7 CFU g^{-1} tissues) at least two orders of magnitude above the concentration of the water (1×10^5 CFU ml^{-1} water) after 2 hours exposure. Within 24 hours of depuration, mussels release concentrated *V. anguillarum* in feces and pseudofeces matter (1.3×10^7 CFU g^{-1} feces). After 72 h of depuration, *V. anguillarum* is no longer detected in the fecal matter. ISAV RNA genome was detected by qRT-PCR in mussel digestive gland tissue after 24 h exposures. However, viable ISAV particles were not detected in mussel digestive gland tissues by cell culture techniques. It is possible that viable particles were present in the tissues but were below the detection limit of our assay. Alternatively, mussels are inactivating ISAV particles and therefore the virus is only detectable by qRT-PCR and not culture techniques.

Objective 2/Milestone 4: Mussel/Pathogen/Fish interaction experiments;
Mussel/Pathogen/Fish experiments were designed and carried out. Juvenile cod exposed to *Vibrio*-loaded mussel feces had significantly decreased survival compared ($p=0.0001$) compared to juvenile cod exposed to control mussel feces. This suggests that mussels could increase the infectious pressure of *V. anguillarum* O2β on IMTA farms culturing cod. A mussel/salmon/ISAV trial was carried out; however we were unable to demonstrate that mussels could potentially decrease the infectious pressure of ISAV on IMTA farms culturing salmon because the trial was prematurely terminated.

Objective 3/Milestone 5: Seasonally placed sentinel mussels at selected finfish sites;
Sentinel mussels were placed out on a salmon farm starting in October 2008. These mussels were collected and new sentinel mussels were placed out on the farm in October 2008, November 2008, March 2009, May 2009, June 2009, July 2009, August 2009 and October 2009. Mussels were sampled for disease using molecular and culture techniques. No disease was detected in the samples by Rutgers or UMaine.

Objective 3/Milestone 6: Placement of sentinel mussels at farms experiencing disease outbreaks;
Work was not completed on this objective because growers reported no disease outbreaks over the period of the project.

Objective 4/Milestone 7: Extension component.
The extension component of this project has been extremely successful and generated a number of presentations throughout the country on this research. There are at least two manuscripts currently being prepared as a result of this work and the work will form a significant portion of the doctoral thesis for Mike Pietrak.

To date the planned workshop has not been held. Given that the research group has been awarded a second grant by NRAC to continue and follow up on the work in this project, the proposed workshop will be more effective and useful for growers if the information from both projects were combined into a single workshop. For this reason the workshop has been postponed until the winter of 2011/2012 when a significant portion of the data will be available from the follow on project.

IMPACTS: In concise statements (possibly a bulleted list) indicate how the project has or will benefit the aquaculture industry either directly or indirectly and resulting economic values gained (where appropriate).

This project has demonstrated that mussels have the potential to reduce the infectious pressure of pathogens when integrated on a fish farm. The direct collaborations with industry and the demonstration of concepts in this proposal have lead directly to further research partnerships. Most critically it has opened the door for a partnership with industry to deploy a commercial scale mussel raft on an existing salmon farm in order to investigate the use of mussels as a management strategy for sealice.

RECOMMENDED FOLLOW-UP ACTIVITIES: State concisely how future studies may be structured.

NRAC is currently funding a follow-up project to investigate the final pathogen physiologies, and to evaluate the economics involved. Future studies beyond the currently funded follow-up project should focus on two primary aspects. First, efforts should focus on pathogen ecology on a farm, in particular microbial interactions with the benthos and fouling communities. Second, the development of predictive grower tools based on the pathogen ecology and local and regional hydrography that would allow growers to enter data about a potential IMTA site and gain a reasonable understanding of the potential risks and benefits associated with a given site or gear configuration.

SUPPORT: Use the format in the table below to indicate NRAC-USDA funding and additional other support, both federal and non-federal, for the project. Indicate the name of the source(s) of other support as a footnote to the table.

YEAR	NRAC-USDA FUNDING	OTHER SUPPORT					TOTAL SUPPORT
		UNIVER-SITY	INDUSTRY	OTHER FEDERAL	OTHER	TOTAL	
1		22,175					22,175
2		27,836					27,836
TOTAL							50,011

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED: List under an appendix with the following subheadings: *Publications in Print*; *Manuscripts*; and *Papers Presented*. For the first two subheadings, include journal articles, popular articles, extension materials, DVDs, technical reports, theses and dissertations, etc. using the format of the Transactions of the American Fisheries Society (example below). Under *Papers Presented* subheading include the authors, title, conference/workshop, location, and date(s).Example of Transactions of the American Fisheries Society citation format: Billington, N., R. J. Barrette, and P. D. N. Hebert. 1992. Management implications of mitochondrial DNA variation in walleye stocks. North American Journal of Fisheries Management 12:276-284.

Appendix 1: PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED

Manuscripts

Pietrak, M., Molloy, S.D., Bouchard, D., Singer, J.T., and Bricknell, I. Potential Role of *Mytilus edulis* as a vector of *Vibrio anguillarum* on an Integrated Multi-Trophic Aquaculture Farm. In preparation.

Molloy, S.D., M.R. Pietrak, D.A. Bouchard, and I. Bricknell. Host pathogen interactions of infectious salmon anemia virus in the blue mussel, *Mytilus edulis*. In preparation.

Papers Presented

Molloy, S.D., M.R. Pietrak, D.A. Bouchard, J.T. Singer, I. Bricknell. Mussels As A Barrier to Infectious Salmon Anemia Transmission in Salmon on Integrated Multi-Trophic Aquaculture Farms. 2010. Eastern Fish Health Workshop, Shepherdstown, WV.

Molloy, S.D., M.R. Pietrak, D.A. Bouchard, J.T. Singer, I. Bricknell. Do mussels help spread or eliminate disease on an integrated multi-trophic aquaculture farm? Aquaculture America 2010. San Diego, CA.

M.R. Pietrak, S.D. Molloy, and I. Bricknell. 2009. Do mussels help spread disease or eliminate it on an integrated poly-trophic fish farm? 34th Eastern Fish Health Workshop, Lake Placid, NY.

M. R. Pietrak, S. Molloy, D. Bouchard J. Singer, and I. Bricknell. Clearance Of A Bacterial Fish Pathogen By Mussels on an IMTA Farms: The scoop on mussel poop. 35th Eastern Fish Health Workshop, Shepherdstown, WV.

M. R. Pietrak, S. Molloy, D. Bouchard J. Singer, and I. Bricknell. Potential for Disease Transmission on an IMTA Farm: Can I add another Species? 1st US IMTA Workshop. Port Angeles, WA

M. R. Pietrak, S. Molloy, D. Bouchard J. Singer, and I. Bricknell. Interaction of a bacterial fish pathogen *Vibrio anguillarum 02β* with mussels *Mytilus edulis*. Northeast Aquaculture Conference and Exposition 2012, Plymouth, MA.

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PART II

TECHNICAL ANALYSIS AND SUMMARY: Describe the work undertaken and results obtained for each objective. Major results should be presented in detail, including graphs, charts, figures, photomicrographs or other presentations. Methodology should be briefly described and statistical analyses and significance should be included where appropriate. This section of the report should be written with style similar to scientific publication. Reports previously or currently prepared for publication may be submitted as part of this section.

PROJECT COMPLETION REPORT

Objective 1/Milestone 1

Work on this Milestone was done by Rutgers and Dr. Molloy at the University of Maine. Progress towards accomplishing this milestone encountered two hurdles. First, issues with contracts delayed Rutgers from being able to begin the work until October. Rutgers was doing most of the work on optimizing the molecular assays for ISAV. In particular they had to develop new housekeeping genes in order to develop quantitative PCR assays. Unfortunately, Dr. Landau who was conducting this work took a new position during this period. She was able to begin the required housekeeping gene screening and optimization work, prior to her departure, but it was not completed before December 31. Despite these setbacks, Dr. Molloy has been able to complete the work. Dr. Molloy designed a primer and probe set that corresponds to the elongation factor 1-alpha sequence in the Blue mussel. Because this primer/probe set was designed to span an intron, amplification with these primers and signal from this probe can only occur from elongation factor 1-alpha messenger RNA. This primer probe set has been optimized to use alongside the ISAV-specific primer/probe set in the real-time RT-PCR assay in order to assess the starting amount of RNA and to normalize the pathogen gene-specific data.

Objective 1/Milestone 2

There are two major goals associated with this milestone. The first is an optimization and standardization of culture techniques to be used in the evaluations. This was successfully accomplished. Staff at the Maine Aquatic Animal Health Lab was trained under the supervision of Debbie Bouchard in the cell culture techniques to be used for virus culture and detection. During this time two batches of virus were cultured in order to provide a single lot of virus sufficient for the planned experiments. A strain of pathogenic *Vibrio anguillarum* 02β carrying a plasmid that encodes an ampicillin resistance gene and a red fluorescent protein gene was acquired from Dr. Singer's lab at the University of Maine. The graduate student was taught the appropriate culture techniques for culturing the bacteria and experimental infection protocols were developed and standardized.

The second major goal of this milestone was to establish standardized procedures for conducting molecular assays on split samples at both Rutgers and Maine and to ensure quality control by having both labs analyze a number of split samples. The establishment of these protocols was difficult to accomplish prior to the completion of assay development in milestone 1. Having said that several meetings were held to begin this process, so that once milestone 1 was completed in February, Dr. Ford and a technician were able to visit Maine and learn how to conduct the ISAV real-time RT-PCR assay and finalize the protocols for split sampling and quality control. These procedures are in place and functioning as designed.

Objective 2/Milestone 3

As reported in the previous project report two experiments were conducted on *Vibrio anguillarum* 02β. These results (Fig 1 and 2) suggested that mussels accumulate viable *V. anguillarum* in their digestive gland tissues and therefore might potentially increase the infectious pressure for *V. anguillarum*. A shedding experiment was conducted to determine if *V. anguillarum*-loaded mussels could shed viable bacteria into the environment either through the water or fecal and pseudo-fecal material (hereafter referred to as fecal matter). This experiment demonstrated that mussels did not shed viable bacteria into the water. Mussels did shed viable *V. anguillarum* in fecal matter for the first 48 hours after being removed from the infected water (Fig. 3). This experiment confirms the potential for mussels to increase the infectious pressure of *V. anguillarum*. Subsequent fish trials were conducted and the results are presented under Milestone 4.

The fate of infectious salmon anemia virus (ISAV) was also determined in mussels. Mussels were exposed to 10^4 $TCID_{50}$ of ISAV per ml and sampled between 2h and 96 h post exposure. ISAV RNA was detected in mussel digestive gland by the quantitative RT-PCR (qRT-PCR) assay optimized in objective 1 (Fig. 4). Viable ISAV, however, was not detected by 50 % tissue culture infective dose ($TCID_{50}$) analysis in Chinook salmon embryo (CHSE) cells in any of the digestive gland samples at any time point. It is possible that viable virus was present in digestive gland tissues at levels below our detection limit ($<10^3$ $TCID_{50}$ ml^{-1}) or the mussel removing particles from the water column and inactivating them. We recently optimized the $TCID_{50}$ assay for use in Atlantic salmon kidney (ASK) cells and demonstrated an increased sensitivity by nearly two orders of magnitude compared to the assay performed with CHSE cells. The mussel ISAV exposure trial will be repeated in January 2011 to confirm that mussels inactivate ISAV. Fish trials were conducted and the results are presented under Milestone 4.

Objective 2/Milestone 4

Based on previous results that indicated mussels had the potential to increase the infectious pressure of *V. anguillarum*, an infection trial was designed to verify if the viable *V. anguillarum* found in mussel fecal matter was capable of causing infection in juvenile cod. Replicate groups of naïve cod were bath-challenged with negative control mussel fecal matter, *Vibrio*-loaded fecal matter (10^5 CFU ml^{-1}), and *Vibrio* alone (10^5 CFU ml^{-1}) as a positive control for 1 h. Dead and moribund fish were removed daily and sampled for culture analysis to verify if vibriosis was the likely cause of death.

Cod challenged with *Vibrio*-loaded mussel fecal matter suffered 58% to 75% mortalities due to vibriosis. There were no mortalities due to vibriosis in fish exposed to control mussel fecal matter (Figure 5). According to Kaplan-Meier analysis, mortalities were significantly greater in cod in the *Vibrio*-loaded feces treatment compared to that in cod in the negative control feces and *V. anguillarum*-alone treatments. Two fish died in the negative control treatment; however *V. anguillarum* was not detected in kidney samples. There was 33% mortality in the positive control fish exposed to *V. anguillarum* alone. Sampling at the end of the trial did not detect *V. anguillarum* in any of the surviving fish regardless of treatment.

An experiment was designed to determine if mussels could decrease mortalities in naïve Atlantic salmon located downstream of ISAV-infected salmon and a mussel barrier compared to naïve fish located downstream of ISAV-infected salmon with no mussel barrier. Atlantic salmon smolts were obtained from a commercial hatchery and transferred to the University of Maine wet lab where they were maintained in recirculating systems containing 10 °C artificial salt water. Each replicate system contained 3 40-gallon tanks with water flowing from Tank 1 to Tank 2 to Tanks 3. Salmon were randomly assigned to Tanks 1 and 3 in each system until each tank contained 40 fish. In two replicate systems, Tank 2 contained 200 mussels. In control system, Tank 2 contained water only. ISAV stock (passaged twice in CHSE cells after isolation from Atlantic salmon) was diluted in PBS. $TCID_{50}$ analysis of the inoculum demonstrated a titer of 2.5×10^4 $TCID_{50}$ ml^{-1}. In all of the replicate systems, 10 % of the salmon from Tank 1 were intraperitoneally (i.p.) injected with 100 µl of the ISAV solution. Fish were observed daily for mortalities and morbidities. Dead and moribund fish were examined for external and internal signs of ISA before harvesting kidney tissues for real-time PCR and cell culture analysis. On a weekly basis, mussel samples from Tank 2 and 1-L water samples were taken from Tank 1- and Tank 2-outlets and analyzed for presence of virus using the molecular- and culture-based methods. ISAV was weakly detected by qRT-PCR methods in some mussel samples but no ISAV was detected in any of the water samples. At 7 weeks post challenge, a system failure caused mortalities in all of the salmon in Tank 1 of the control systems. Therefore the experiment was stopped and all of the salmon in Tanks 1 and 3 of each system were euthanized and analyzed for presence of virus using molecular- and culture-based techniques. Because the experiment ended before mortalities began in the ISAV-naïve salmon in Tanks 3, we had hoped to see a significant difference in the number of ISAV-positive fish between the mussel treatment and control treatment. Very few of the fish in Tank 3, regardless of treatment, were ISAV-positive and there was no significant difference between treatments. The results of our qPCR and cell culture analyses, however, were in complete agreement, although the qPCR detection method was more sensitive.

Objective 3/Milestone 5: Seasonally placed sentinel mussels at selected finfish sites; Sentinel mussels were placed out on a salmon farm starting in October 2008. These mussels were collected and new sentinel mussels were placed out on the farm in October 2008, November 2008, March 2009, May 2009, June 2009, July 2009, August 2009 and October 2009. Once the site diver collected the mussels, 36 mussels from each of the

five sentinel bags were randomly selected and sampled for disease using molecular and culture techniques. Molecular samples were processed by Rutgers University with quality control being conducted by the University of Maine. No disease was detected in the samples.

Objective 3/Milestone 6: Placement of sentinel mussels at farms experiencing disease outbreaks;
Work was not completed on this objective because growers reported no disease outbreaks over the period of the project.

Objective 4/Milestone 7: Extension component.
The extension component of this project has been extremely successful and generated a number of presentations throughout the country on this research. These presentations are listed above in this report. There are at least two manuscripts currently being prepared as a result of this work and the work will form a significant portion of the doctoral thesis for Mike Pietrak.

To date the planned workshop has not been held. Given that the research group has been awarded a second grant by NRAC to continue and follow up on the work in this project, it was decided that the proposed workshop would be more effective and useful for growers if the information from both projects were combined into a single workshop. For this reason the workshop has been postponed until the winter of 2011/2012 when a significant portion of the data will be available from the follow on project. This timeframe also coincides with the slow part of the growing season for farmers so that participation can be maximized.

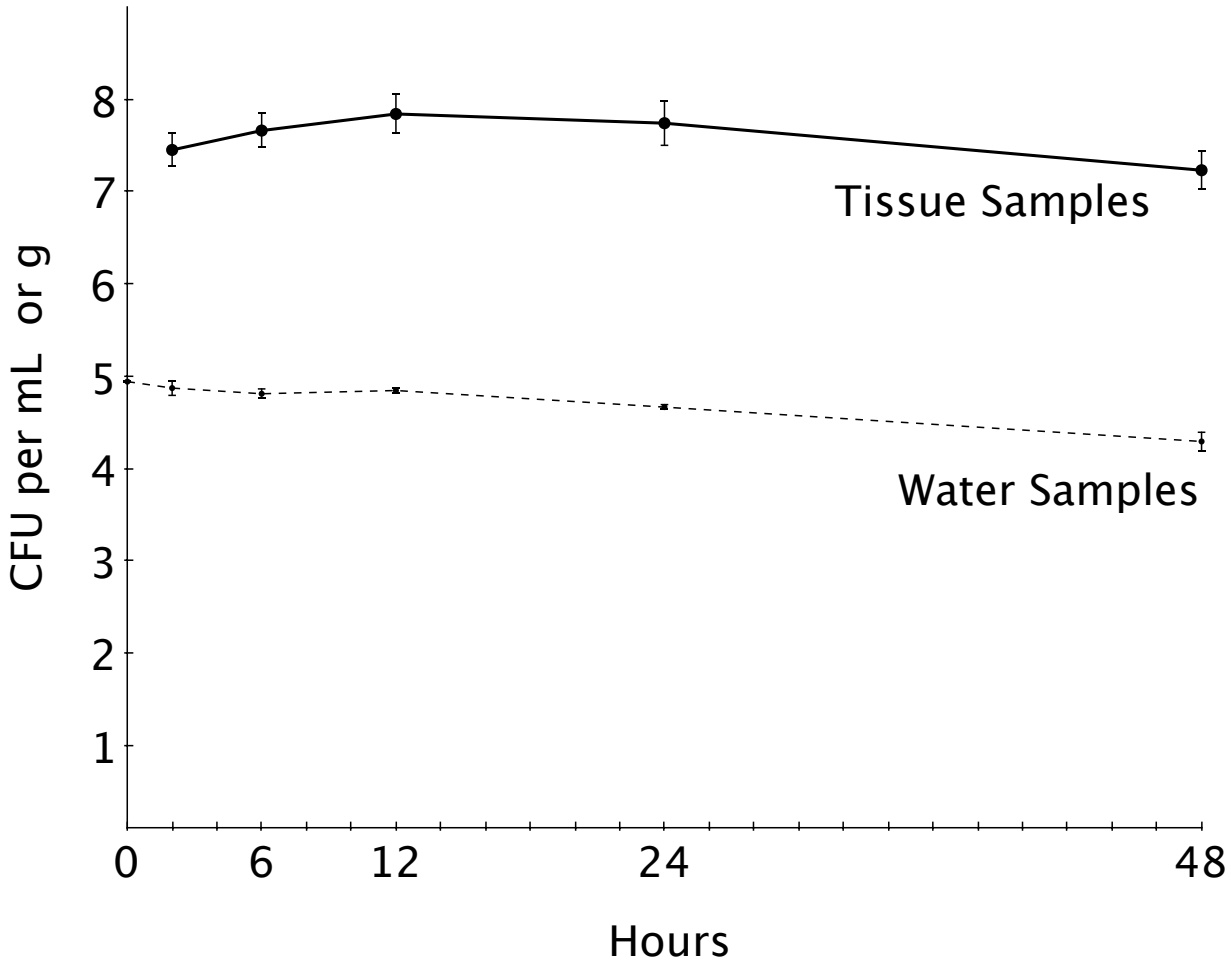

Figure 1. The average colony forming units (CFU) of *V. anguillarum* per ml of water or per gram of digestive gland tissue ± SE (n=9 per time point) from three replicate tanks of mussels exposed to 10^5 CFU ml^{-1} *V. anguillarum* for 48 h. The dashed line represents the average CFU mL^{-1} of water ± SE (n=3 per time point). There was a significant difference in the amount of *V. anguillarum* detected between the digestive gland and the water ($p<0.05$)

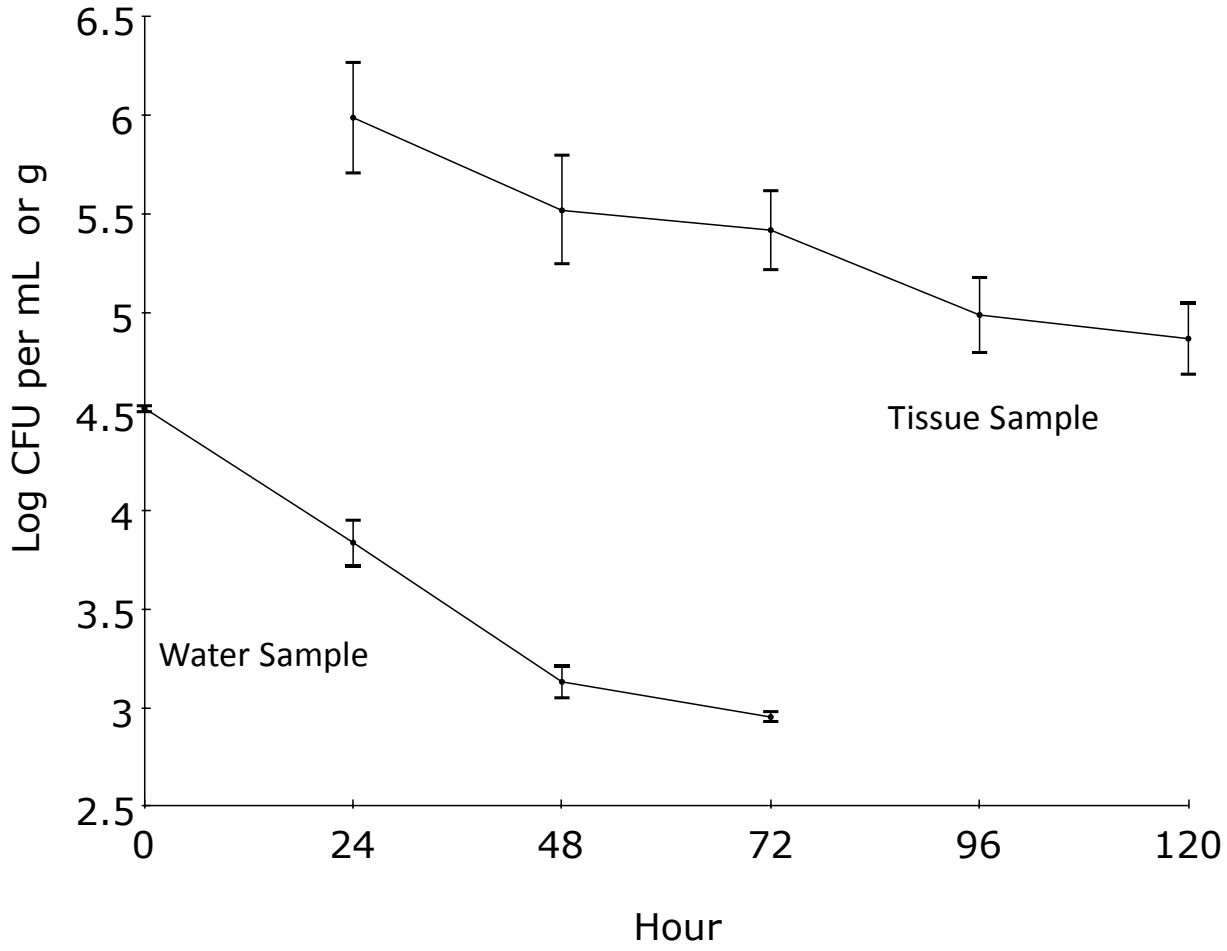

Figure 2. The average CFU of *V. anguillarum* per ml of water or per gram of digestive gland tissue ± SE (n=9 per time point) from three replicate tanks of mussels exposed to 10^5 CFU ml^{-1} *V. anguillarum* for 120 h. The dashed line represents the average CFU mL^{-1} of water ± SE (n=3 per time point). There was a significant difference in the amount of *V. anguillarum* detected between the digestive gland and the water ($p<0.05$)

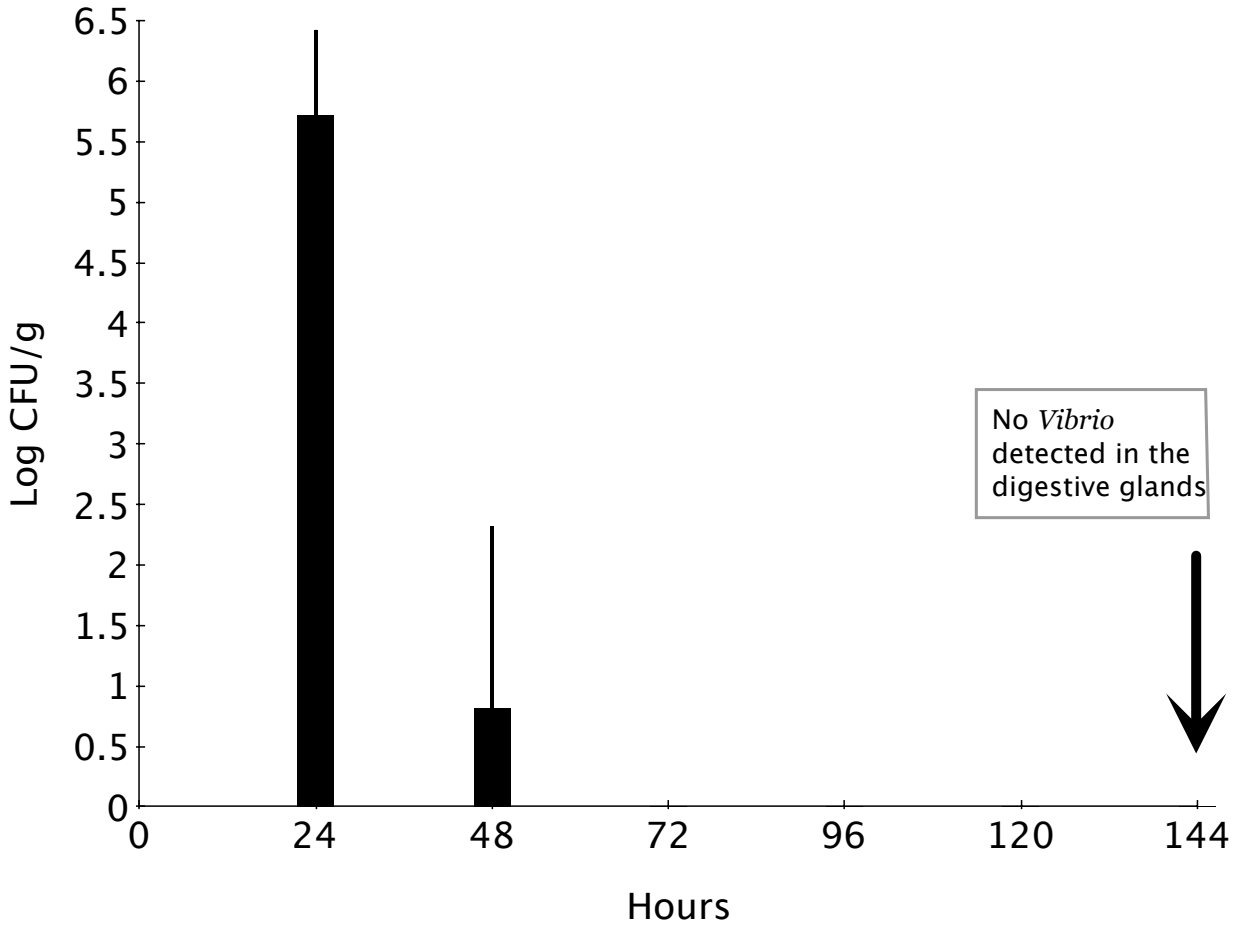

Figure 3. The average CFU of *V. anguillarum* 02β per gram of feces ± SE (n=8). After 48 hours, only 2 of the 8 mussels shed levels of V. anguillarum high enough to be counted. *V. anguillarum* was not detected in the water samples.

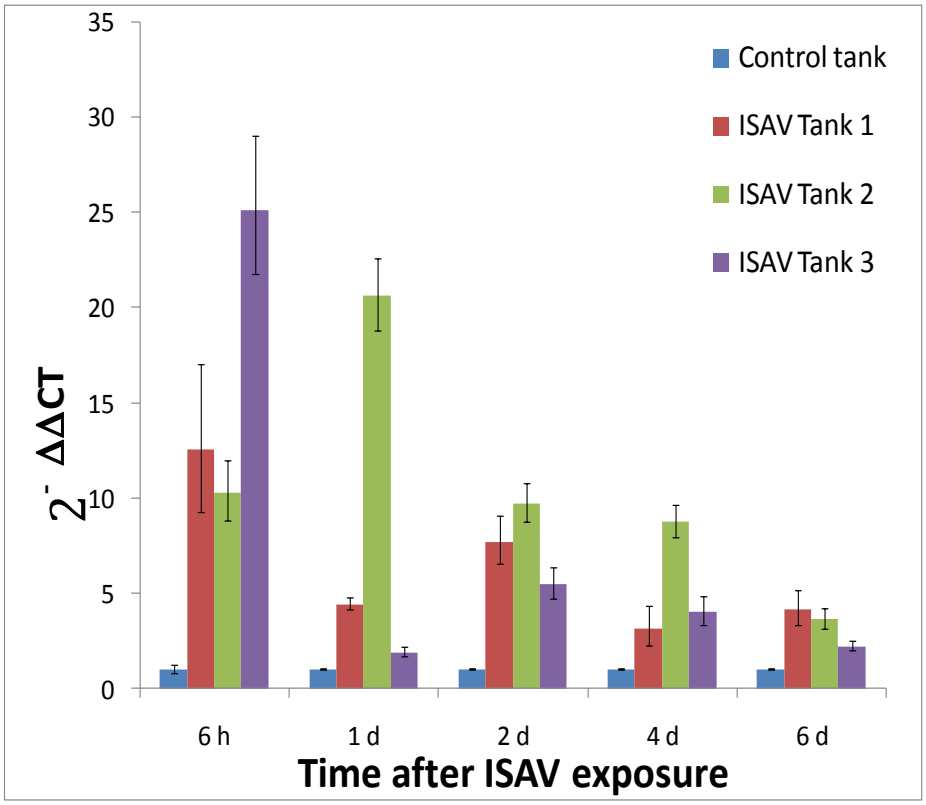

Figure 4. ISAV segment 8 relative abundance in mussel digestive glands at 6 h, 1 d, 2 d, 4 d, and 6 d after exposure to ISAV as measured with Taqman quantitative RT-PCR. Graphs represent average values ± standard error of the mean with n=3.

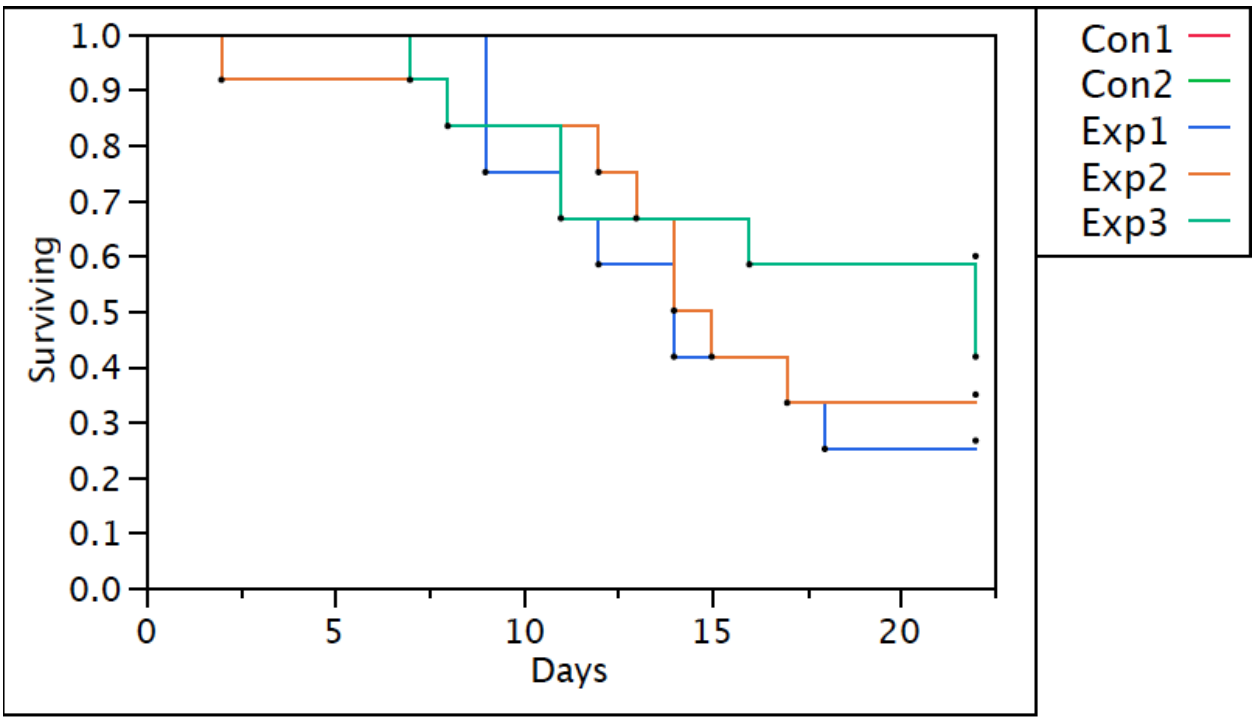

Figure 5. Kaplan meier analysis of cod survival after a 1 h exposure to *V. anguillarum*-loaded mussel feces and control mussel feces. Cod exposed to control mussel feces (Con1 and Con2) had 100 % survival. Cod exposed to *V. anguillarum*-loaded mussel feces (Exp1, Exp2, and Exp 3) had percent survivals ranging from 13.5 to 18.2. Cod exposed to control mussel feces had significantly greater survival compared to cod exposed to *V. anguillarum*-loaded mussel feces (p=0.0001).