Today’s date: 3-3-14

Project number: RI/SSS-6

Project title: Reducing the Extent of Permanently Closed Shellfish Growing Areas through Regulatory Modernization: Enteric Virus Contamination and Mitigation Strategies

Project initiation date: 9/1/2012

Project completion date: 8/31/14

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Partner(s) and affiliation(s) with SCALE (local, state, regional, national, international) and TYPE (gov’t, NGO, industry/business, academic institution, other) (List any collaborators, sponsors, industry partners, municipalities, etc., associated with this project):
Kohl Kanwit, Maine Dept. of Marine Resources; regional; government
Michael Hickey & Jeff Kennedy, Mass. Division of Marine Fisheries; regional; government
Kristin DeRosia-Banick, CT Dept. of Agriculture; regional; government

Brief project overview/Abstract:
In the Northeastern United States, many acres of productive shellfish growing waters are permanently closed to aquaculture and harvesting activities due to concerns associated with human fecal pollution from municipal wastewater treatment plant (WWTP) outfalls. Often, these potential aquaculture sites are highly productive, sheltered, near-shore locations with good commercial access. The main Outcome for this project is to increase coastal areas that are permitted for shellfish growing and harvesting. The size of these areas is based in part on FC
levels, yet scientific investigations have demonstrated that FC levels are often unrelated to enteric virus occurrence and are misleading with respect to virus contamination. Norovirus (NoV) is responsible for the majority of shellfish consumption related viral disease worldwide with significant health costs in the US. Male-specific coliphage (MSC), a bacteriophage of E. coli bacteria and a small, round, RNA virus like NoV, has been proposed as a specialty viral indicator organism that better reflects the persistence of viruses in molluscan shellfish meats impacted by wastewater. The proposed approach is a well-defined, comprehensive approach to determining the feasibility of expanding aquaculture and harvest of hard clams and oysters in the Northeast US. The approach involves determining seasonal environmental conditions for minimal enteric virus contamination, and the effectiveness of seasonal MSC relay and depuration for purging of low-level microbial contamination. Highly similar previous studies in Maine with soft-shelled clams lead to successful re-opening of closed, highly productive areas with significant local economic benefits. This project has a high probability of success in leading to changes in the National Shellfish Sanitation Program and increasing available growing areas in New England and nationally. The project will also positively impact aquaculture development, quality assurance processes, and ultimately consumer confidence, thus successfully achieving our overall Outcome.

Objectives:
The overall project goal is to open currently closed, yet highly productive, shellfish beds for aquaculture and harvest based on solid scientific evidence ensuring shellfish safety. The primary research objective is to determine the efficacy of using male-specific coliphage (MSC) as a model indicator of enteric viral contamination for Eastern oysters and hard-shelled clams (quahogs) and for assessing relay and depuration viral removal kinetics. The specific objectives are as follows:

1. Determine the relationships between water temperature-influenced seasonal levels of norovirus (NoV), MSC and fecal coliforms (FC) in Eastern oysters (Crassostrea virginica) and hard-shelled clams (Mercenaria mercenaria) harvested from a mid/southerly New England estuarine system such as the Taunton River-Mount Hope Bay estuarine system, MA;
2. Determine relative NoV, MSC and FC elimination (reduction) kinetics during relay and depuration as they relate to water temperature and season.

Research findings/progress during 2/1/13 – 1/31/14:
Single Lab Validation Study
A Single Lab Validation (SLV) for detecting MSC levels in quahogs is a necessary step to provide regulatory and private labs a key tool needed to help alleviate barriers to quahog aquaculture and harvesting. The quahog SLV conducted by Spinney Creek Shellfish, Inc. (SCS) has been completed. Three members of the of the Massachusetts DMF laboratory staff were involved with the SLV testing on some of the required test runs. Ten trial batches were analyzed using quahogs from ME, CT, NJ and VA. The limit of detection was 5 plaque-forming units (PFU)/100 g and the limit of quantitation/sensitivity was 47 PFU/g. The results were compiled, analyzed, summarized in a report and submitted to the ISSC Lab Methods Review Committee. At the Biennial Meeting of the ISSC held in January 2014 the method was approved as a method for use in detecting MSC in hard clams.

Seasonal Field Studies
Our study goals also included determining seasonal levels of MSC and NoV in hard-shelled clams and Eastern oysters from southern New England waters. The Taunton River in Massachusetts was recommended as the main study site for this fieldwork. Oyster and quahog sites were located at Conspiracy Island and Whale Rock in close proximity to each other well
upriver of the Fall River and Somerset WWTP outfalls and downriver of the Taunton WWTP outfall. Soft-shell clams were located adjacent to the Somerset outfall. We established a monitoring program that involved collecting monthly shellfish samples from these sites for MSC, NV, AdV and FC analyses. From October 15, 2012 to June 6, 2013, sampling occurred each month except for March 2013, for a total of seven sampling dates.

The Taunton River has not proven to be a useful site for our study goals because of consistently low levels of sewage-borne microbial contaminants in water and both oysters and quahogs. Upon review of the most recent water quality data, the fecal coliform levels in water were relatively low; the geometric mean for all water samples was 39.6 FC/100 ml. MSC were detected (just above detection limits) in 15 of 16 shellfish samples and in 13 of 16 water samples. The MSC concentrations in water, quahogs and oysters, however, were relatively low. The geometric mean concentrations were 18, 18.4 and 22.2 PFU/100 g for oysters, quahogs and water, respectively. The MSC levels were too low for depuration and relay experiments, the other main goal of this research study. The significant difference in MSC levels between shellfish species is a potentially important observation, and will be the focus of research moving forward (see below).

Norovirus (NoV GII) and adenovirus (AdV) were also present at low levels in shellfish samples from the Taunton River. NoV G1 was not detected in any of the 10 samples analyzed to date, while NoV GII and AdV were detected in 6 and 4 of the 10 samples, respectively. The highest NoV GII concentration was 12.9 PCR units (U)/g in quahogs and the highest AdV concentration was 52.5 U/g in soft shell clams.

There was no relationship between FC and MSC/virus levels. The FC concentrations varied to a greater degree than the virus concentrations, but the levels of each did not track temporal trends well; when FC concentrations spiked, the MSC and virus levels typically did not coincidentally change.

The apparent effectiveness of the WWTPs along the Taunton River is good news and suggests potential management and classification options, consistent with the overriding goal for this research, i.e., “overcome regulatory hurdles” for shellfish harvesting. The low levels of microbial contaminants, however, preclude this area as a useful study site for this project. A new study area from among several potential areas was chosen for continuing research for this study (see below).

Studies conducted in the Taunton River showed relatively low MSC levels and accumulation rates compared to soft-shelled clams. MSC and FC levels were too low to see any seasonal persistence patterns in oysters and quahogs, and MSC in soft-shelled clams harvested close to the Fall River and Somerset outfall were not particularly high. For these reasons, we concluded that the Taunton River is really not all that contaminated. This is based on the findings from this study and other recent water testing that seems to confirm that 30 years of public investment in treatment plant upgrades and watershed management efforts have resulted in marked water quality and environment improvement.

After this initial work in the Taunton River in Massachusetts showed that levels of sewage borne microbial contaminants were too low for this study, we focused on two new sites. After in-depth discussions with MA DMF co-investigators and their staff, we chose the Salem Sound area because it has a significant secondary sewage treatment facility, the South Essex Sewage District (SESD) facility designed for a 41 MGD discharge, and abundant shellfish resources. The other site is called ‘Three Rivers’ and is located at the junction of the upper Piscataqua, Cochecho and Salmon Falls rivers in ME/NH. This area is impacted by WWTP discharges upstream in the Salmon Falls River and downstream in the Piscataqua River. The combination
of these two study areas allows for seasonal intercomparison studies of virus uptake and depuration in quahogs and European oysters compared to soft shell clams in the Salem Sound area, and between American oysters and soft shell clams at the Three Rivers area.

A number of sites in the Salem Sound were assessed as possible study sites on August 22, 2013. As part of this survey, we sampled soft shell clams, European oysters and water from four different areas, including Bass River, Collins Cove, Forest River Park and Marblehead Harbor. Based on findings from these screening analyses and review of other existing data, including information on prevailing currents, initial experiments were then set up to investigate species-specific differences in bio-accumulation using soft-shelled clams as a control species. Soft-shelled clams were proposed as a control species because in previous intensive studies, their seasonal persistence and temperature/seasonal depuration rates had been well established. The specific study sites and shellfish species were:

- Marblehead Harbor – Clean quahogs from the Taunton River were transplanted in close proximity to native soft-shelled clams
- Salem Harbor – Native soft-shelled clams, European oysters were present
- Three Rivers – Native American oysters and soft-shelled clams
- Collins Cove in Danvers River – Native soft-shelled clams

The selected shellfish species and water were sampled at each site at approximate monthly sample dates and samples were analyzed for MSC, FC and enteric viruses (NoV and AdV). Water samples were also collected at a large stormwater pipe discharging into Marblehead Harbor.

Norovirus (NoV GI & GII) and adenovirus (AdV) were also present at low levels in shellfish samples from Marblehead Harbor and Three Rivers. NoV GI was detected in 3 of 12 total shellfish samples, including in one of 4 American oyster and soft shell clam samples from Three Rivers and from one of 3 soft shell clam samples from Marblehead Harbor. NoV GII was detected in 4 of 12 total shellfish samples, including in 3 of 4 clam samples from Three Rivers and in 1 of 3 clam samples from Marblehead. AdV was detected in 9 of 12 total shellfish samples, including in 3 of 4 oyster and all 4 soft shell clam samples from Three Rivers, as well as in one sample each of clams and quahogs from Marblehead Harbor. The highest NoV GI concentration was 8.3 PCR units (U)/g in American oysters from Three Rivers, the highest NoV GII concentration was 21.3 PCR units (U)/g in softshell clams from Three Rivers, and the highest AdV concentration was 76.5 U/g in soft shell clams from Three Rivers. NoV GII and AdV levels increased with time as water temperatures became colder.

Similar to what was found in the Taunton River, there was no relationship between FC and MSC/virus levels at the two new study sites,. The FC concentrations varied to a greater degree than the virus concentrations, but the levels of each did not track temporal trends well; when FC concentrations spiked, the MSC and virus levels typically did not coincidentally change.

This study has helped to deepen the understanding of the seasonality of MSC in shellfish, especially using native soft shell clams. The findings have been added to findings from previous studies in Maine to provide an extensive database for this species at 4 sites in Maine (Royal, Fore and Presumpscot rivers & Three Rivers) and 5 sites in MA, 3 in the Salem Sound area (Marblehead Harbor, Collins Cove, Forest River Park) and 2 in the Taunton River area (Somerset, Tugboat). There are several important trends that are revealed in these data (Figure 1). There is a distinct seasonal trend for MSC in soft shell clams where the lowest levels are detected in June-July and the highest levels in December-January, closely tracking water temperatures. The small amount of data available through this study for European oysters,
American oysters and quahogs suggests the European oysters exhibited a similar seasonal MSC trend as the soft shell clams, while American oysters and quahogs did not. The other trend is the levels at specific sites reflect the level of contamination present, where the highest levels were detected in the Fore River, Maine that is impacted by the nearby South Portland WWTP and multiple CSO's while lower levels were detected at sites impacted by smaller WWTPs or where the WWTP discharge was more distant.

The main conclusions for this project to date are:

- 'Cold-water adapted' species such as soft-shelled clams, Pacific oysters, and European oysters demonstrate strong seasonal MSC patterns.
- Non cold-water adapted species such as American oysters and quahogs are anomalous because they apparently stop pumping at water temperatures below 10°C and do not reflect levels of MSC and viruses in the water column.
- American oysters and quahogs have demonstrated similar seasonal MSC levels as cold-water adapted species in other related studies in southern waters where water temperatures do not drop below 10°C.
- American oysters and quahogs can trap MSC for the winter months if significant MSC contamination is present in the environment prior to when water temperature falls below 10°C, and these levels do not significantly change until water temperatures increase and pumping is re-initiated in spring.
- FC indicator bacteria apparently die-off when American oysters and quahogs stop pumping over the winter period, as they exhibit overall declines in concentration through the winter season, in contrast to MSC levels.

Building Collaborative Studies with Different States
We had a productive exchange of information with the State of NJ concerning their general experience with quahogs, their Hurricane Sandy experience in using MSC to re-open harvest areas, and our SLV and soft-shelled clam work. NJ has in-depth post-Sandy data for MSC in quahogs. Results from Marblehead with transplanted quahogs showed consistency with data collected by NJ after Hurricane Sandy, demonstrating the process of trapping viral particules for winter months. We also met with Washington state regulators, scientists and industry to exchange information and share our experience with MSC. Both Washington and New Jersey state programs showed interest in conducting collaborative studies that use MSC as an indicator of viral contamination while determining the sanitary conditions of shellfish growing areas. Ongoing dialogue is focused on logistics for doing that.

Research Findings Significance
The significance of these findings for changing policies that currently limit shellfish harvesting are broad reaching and timely. The results of the research findings to date, in collaboration with state agencies, industry and the FDA, have been instrumental in furthering the progress of key shellfish aquaculture policies under consideration by the ISSC. There are several key policies that relate to the use of MSC for expanding areas and time for harvest opportunities that previously had been delayed due to concerns about the lack of information on MSC as an indicator of enteric virus contamination for shellfish harvest management. In February 2012, FDA characterized these information gaps under 4 topics of study: 1) the uptake and elimination of NoV, enterovirus, and MSC by shellfish species other than soft-shelled clams. These investigations should be conducted in multiple geographic locations representative of the country and over all seasons. 2) A SLV is needed to demonstrate the efficacy of this or another method to enumerate MSC in species of shellfish other than soft-shelled clams and oysters. 3) Examine and compare MSC and enteric virus levels in wastewater influent and effluent, shellfish receiving waters, and shellfish. 4) Understanding the efficiency of various wastewater treatment
systems to inactivate/remove enteric viruses prior to discharge. The first three of these are directly addressed by this study, while the FDA is undertaking the fourth study topic.

The combined findings from this study and studies by FDA since February 2012 are serving as the basis for the ISSC Growing Area Committee to re-write several (see below) ISSC proposals that will incorporate MSC into shellfish management policies and be in place to be reconsidered for adoption by fall, 2015. The study findings have also directly instigated a regulatory action— a MSC Summit organized by the ISSC to be held this year to review all scientific knowledge about enteric viruses-MSC-shellfish contamination.

Previous studies have reported vaguely defined differences between shellfish species in relation to uptake and depuration of MSC and viruses. These observations have fueled uncertainty about the utility of MSC as a viral indicator, and lead to hesitation in bringing MSC into use for shellfish harvest management. The findings for this study so far have made great progress toward defining the actual differences and effectively addressing lingering uncertainty about MSC.

Finally, the findings of this study have helped to provide FDA the scientific knowledge to further their development of using MSC as a modeling organism to determine WWTP performance with respect to viruses. Co-Investigator Howell is currently assisting FDA in modifying their MSC method for wastewater influent and effluent and in performing method validation work (SLV) to be submitted to the ISSC Laboratory Methods Review Committee. The findings for this study and related work are also support discussions between FDA and EPA pertaining to improving WWTP performance, which would have the broad impact of improving water quality in proximity to WWTPs nationwide.

Accomplishments during 2/1/13 – 1/31/14 (Accomplishments are the key actions, activities or products resulting from Sea Grant research projects. They are distinct from impacts in that they reflect ongoing activities or key results that may not yet have had a significant economic, societal and/or environmental benefit but lay the foundation for such a benefit. Accomplishments may evolve into impacts in the future.)

The significance of these findings is broad. Generally, this study helps to define seasonal conditions and shellfish species differences for managing shellfish harvesting in areas affected by WWTP effluent due to proximity to discharge pipes or impacts from WWTP performance failure. These findings further the growing knowledge basis for using MSC as an indicator of viral contamination and the ineffectiveness of fecal coliform for the same function.

The significance of these findings for changing policies that currently limit shellfish harvesting are also broad reaching. The results of the research findings to date, in collaboration with state agencies, industry and the FDA, has been instrumental in furthering the progress of key shellfish aquaculture policies under consideration by the ISSC. There are several key policies that relate to the use of MSC for expanding areas and time for harvest opportunities that are currently limited due to concerns about enteric virus contamination. Several of these policies are in the process of being addressed by the ISSC, as follows:

Proposal #-Title
#11-101-"Re-opening conditional areas using male-specific coliphage after WTP malfunction"
#11-102-"Using male-specific coliphage as a tool to refine determinations of the size of the areas to be classified as prohibited adjacent to each outfall"
Proposals 11-101, 102, and 103 were first reviewed at the 2011 ISSC meeting. As a result the Conference requested FDA to provide more information (data and analysis) to inform deliberations. On February 26, 2012, FDA provided input that there was insufficient data available to make sound science decisions regarding the use of MSC and the need for more scientific study results for use of this tool to manage shellfish harvest areas. They went on to delineate the four main areas of research (see above) needed. This project is one of the main sources of information addressing three of the four research topics, and FDA is taking on the fourth, in collaboration with our project. At the January, 2014 Biennial meeting of the ISSC, the Conference considered some of the new data from this project and made the commitment to have an expert Working Group hold quarterly meetings and to organize an "MSC Summit" where experts will present and discuss the latest knowledge, science, and potential applications for MSC. Investigators on this project will be part of the working group and the summit.

State agencies in NJ, RI and CT have been using MSC as an indicator of viral contamination for several years. Our project team has met with state agency representatives from NJ and CT and state agencies in WA state to compare results and methods to help all involved understand its potential utility as a shellfish harvest management tool. We have consulted with the State of CT on their plans to use MSC as a viral indicator of sewage contamination for their plans to reclassify sections of the Housatonic River from prohibited to restricted for relay. The Housatonic River is a historically productive and important seed source for CT growers. We have ongoing discussions with NH DES shellfish program to test blue mussels (Mytilus edulis) from both local aquaculture sites and relay sites for MSC and viruses.

The study area has been expanded to include Salem Harbor in MA and 'Three Rivers' area of the Piscataqua/Cocheco/Salmon Falls rivers in NH/ME. We have also included analysis of European oysters (Ostrea edulis) as part of the Salem Harbor study. The study now has expanded the number of species being studied from the proposed 2 species to 4 species, as soft shell clams have been adopted as the 'control' species.

We have found that different bivalve shellfish species have different behavior relative to accumulation and depuration of MSC and viruses during cold (<10°C) water conditions. Whereas soft shell clams were an ideal species to study because they continue to pump water throughout the year, American oysters and quahogs appear to shut down when water temperature drops below ~10°C. This condition complicates our ability to test their depuration kinetics during cold conditions. This also has significant implications about the potential to re-open after WTP failures or to even close in response to these events. These results help to confirm what NJ observed with quahogs in some areas following Hurricane Sandy, where some quahogs continued to contain elevated levels of MSC as long as 6 months after the hurricane and a subsequent cold weather event that dropped water temperatures to below 10°C.

Impacts during 2/1/13 – 1/31/14 (Impacts are significant economic, societal and/or environmental benefits of research.):

NOTE: Include quantitative data to validate the impact, if possible.

Proposal 13-120-L, "Male-specific coliphage method for quahogs (M. mercenaria)", was adopted by the ISSC. This provides one of the key means for changing policies for using MSC as a tool to help manage shellfish growing areas and expand harvest opportunities for shellfish aquaculture. This method is a key addition to previously adopted methods for soft shell clams and American oysters.
Four proposals to the ISSC for bringing MSC into the NSSP as an indicator of viral contamination are currently being worked on by the ISSC Growing Area Committee. The re-written proposals will be considered for adoption at the next biennial ISSC meeting in Fall 2015.

As a follow-on to our project team collecting water and shellfish samples in the Taunton River, Massachusetts and Rhode Island are currently undertaking dilution studies with FDA at the Fall River and Somerset WWTP’s. This cumulative work may allow the states to re-classify the area from prohibited to a classification that would allow seasonal harvesting and utilization of abundant shellfish resources.

State lab personnel in MA have been trained to run the MSC detection method for quahogs and are currently seeking certification to run those analyses for regulatory purposes.

**Economic benefits realized during 2/1/13 – 1/31/14** (businesses retained or created, jobs retained or created; market and non-market economic benefits):

Note: Please quantify and provide supporting data if possible.

Employment of laboratory technicians at SCS has been increased through this project. The research findings help to justify ongoing and future employment of not only lab technicians but many more shellfish harvesters in Maine as new harvesting opportunities are being made available through the state shellfish program.

**Tools, technologies or information services resulting from this project that were developed or used during 2/1/13 – 1/31/14 to improve ecosystem-based management (e.g., that reduce contaminants that harm coastal ecosystems and seafood consumers; that track changes in ecosystem processes, biological responses and conditions):**

Proposal 13-120-L, "Male-specific coliphage method for quahogs (M. mercenaria)", has been adopted by the ISSC and will be included in the NSSP Guide Section IV Guidance Documents Chapter II Growing Areas .11 Approved Limited Use Methods for Microbiological Testing, along with previously adopted methods for soft shell clams and American oysters. The use of MSC as an indicator of viral contamination in shellfish is being adopted by the ISSC and incorporated into the NSSP as a key policy and scientific tool for managing shellfish harvesting.

**Patents:**

**Technology transfer** (Has a private company utilized this research successfully?):

- State lab personnel in MA have been trained to run the MSC detection method for quahogs and are currently seeking FDA certification to run those analyses for regulatory purposes.
- Project investigators have traveled to NJ, WA and CT to share project findings and knowledge as these states consider use of MSC as a tool for shellfish harvest management.

**Related grants and contracts** (Other grants and contracts that funded this research or that were obtained as a result of this research):

**Leveraged funding** (leveraged funding comes from outside sources and is used to accomplish the goals and objectives of your project. Match associated with your project is not leveraged funding). Provide amount, source, purpose, and start and end date.

The FDA lab at Dauphin Island, AL has provided in-kind analyses for norovirus and adenovirus on all samples analyzed for MSC to help establish the relationship between concentrations of the indicator-MSC with actual public health threats, the two enteric viruses.

Problems encountered:
Studies conducted in the Taunton River showed relatively low MSC levels and accumulation rates compared to soft-shelled clams. MSC and FC levels were too low to see any seasonal persistence patterns in oysters and quahogs, and MSC in soft-shelled clams harvested close to the Fall River and Somerset outfall were also not particularly high. For these reasons, we concluded that the Taunton River had evels of sewage borne microbial contaminants that were too low for this study. After in-depth discussions with MA DMF co-investigators and their staff, we instead chose the Salem Sound as one of our two new study areas because it has a significant secondary sewage treatment facility, the South Essex Sewerage District (SESD) facility designed for a 41 MGD discharge, and abundant shellfish resources. The other new study areas is called 'Three Rivers' and is located at the junction of the upper Piscataqua, Cocheco and Salmon Falls rivers in ME/NH. This area is impacted by WWTF discharges upstream in the Salmon Falls River and downstream in the Piscataqua River. The combination of these two study areas allows for seasonal intercomparison studies of virus uptake and depuration in quahogs and European oysters compared to soft shell clams in the Salem Sound area, and between American oysters and soft shell clams at the Three Rivers area.

Publications to date (please cite and attach PDF or send a hardcopy, or provide status if not yet published):

   Peer reviewed publications:
   none

   Theses/Dissertations:
   none

   Other communications products (non peer-reviewed pubs, manuals, tech reports, videos, etc.):
   none, aside from several PowerPoint presentations

Presentations to date, with published abstract citation if applicable:
NOTE: For presentations to civic groups, etc. (i.e., to the public rather than a scientific conference), please include number of attendees.
January 30, 2014. Presentation of project findings and prospects for adoption as shellfish harvest management policies before the full delegation at the Interstate Shellfish Sanitation Committee biennial meeting.
January 29, 2014. Presentation and discussion of project findings at the Interstate Shellfish Sanitation Committee biennial meeting with regulators and growers from around the US.

Awards:
none.

Additional information:
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<th>Student Name</th>
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<td>Meghan Hartwick</td>
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<td>Molecular, Cellular and Biomedical Sciences</td>
<td>November 2013 to present</td>
<td>Supplies, travel</td>
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