Oyster Culture and Harvest Practices to Reduce Pathogenic *Vibrio parahaemolyticus* Concentrations in the Northeast US

Stephen Jones, Cheryl Whistler, Randi Foxall, Kristen DeRosia-Banick, Christopher Schillaci, Christopher Nash, Meghan Hartwick, Emily Van Gulick

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INTRODUCTION

This report details study results, accomplishments and outcomes. These are organized in the following major sections:

1. A compilation of existing relevant data to inform study design and update local and regional *V. parahaemolyticus* risk assessments, focusing on regional differences and similarities in husbandry practices, local/regional environmental conditions and disease incidence.
2. Detailed descriptions of the overall approach and state-specific approaches used in each phase and for each state.
3. Written documentation of statistically supported findings, recommendations for improving risk assessments for *V. parahaemolyticus* in shellfish in the Northeast, and how the approaches used can be transferred to other shellfish producing areas of the US.

Vibrio parahaemolyticus is the leading cause of seafood borne illness in the United States (CDC 2015). Pathogenic vibrios were the only major bacterial pathogen that showed increasing relative incidence of illnesses in the US from 1996-2012 (Gillis et al. 2013), while Newton et al. (2012) confirmed this was true for *Vibrio vulnificus*, *Vibrio cholerae* and *V. parahaemolyticus,* concluding that "current prevention efforts have failed to prevent increasing rates of vibriosis". Based on state health department and CDC reports, the annual number of reported human Vibrio infections in the New England region dramatically increased during this time period, especially in Massachusetts and Connecticut where there were a combined 5 cases in 2000 compared to 147 cases in 2013 (Urquhart et al. 2015).

Vibrio parahaemolyticus remains a concern because it had the highest increase in illness incidence rate among pathogenic bacteria for 2018 relative to 2015-2017 (109%) in ten states including Connecticut (Tack et al. 2019). *V. parahaemolyticus* illnesses have been increasing in the New England region due to a variety of factors including increased sea surface temperatures due to climate change (Xu et al. 2015), increased consumption of raw shellfish in the summer when *V. parahaemolyticus* is prevalent, and the establishment of introduced pathogenic *V. parahaemolyticus* strains (Baker-Austin et al. 2013, 2017; Martinez-Urtaza et al. 2012; Urquhart et al. 2016; Whistler et al. 2015; Xu et al. 2015, 2017). Water temperature, salinity, pH and other environmental characteristics can also have significant impacts on *V. parahaemolyticus* prevalence (Takemura et al. 2014; Urquhart et al. 2016; Hartwick et al. 2019).

Common oyster culture and harvest practices in the region can involve the exposure of shellfish to ambient air for various periods of time. For example, following harvest, oysters require varying levels of sorting and cleaning prior to being placed under temperature control. In addition, air-drying is commonly used to remove fouling organisms from oysters and equipment in cage culture operations. During warm conditions, these activities may stimulate the growth of *V. parahaemolyticus* within oysters and differentially increase the absolute and relative abundance of pathogenic strains as compared to pre-exposure, or ambient levels of *V. parahaemolyticus* (1-5). Understanding two key practices:

a.) the extent that pathogenic *V. parahaemolyticus* concentrations increase in oysters following harvest before temperature control, and

b.) the extent that pathogenic *V. parahaemolyticus* concentrations increase in oysters following air-drying of pre-harvest oysters following air-drying of pre-harvest oysters for different durations that is coupled to the length of time required for concentrations in re-submerged oysters to return to background levels (recovery period), is critical to continued industry growth while maintaining product safety.

Several previous studies have provided useful information related to the ambient concentrations of potentially pathogenic *V. parahaemolyticus* in oysters relative to in situ water temperature, water depth, sub- versus inter-tidal culture, the post-harvest *V. parahaemolyticus* growth in oysters over various temperature control strategies. Studies have also looked at the extent of *V. parahaemolyticus* growth in oysters following air-drying during warm months over various exposure periods and the time required for excess *V. parahaemolyticus* levels in re-submerged oysters to return to background levels (Kinsey et al. 2015; Kennedy et al. 2015; Phuvasate et a. 2015; Jones et al. 2016; Suhrbier et al. 2017). These studies have shown mixed results, that suggest the impact of post-harvest exposure on *V. parahaemolyticus* growth, and the time needed to purge excess *V. parahaemolyticus* levels following extended pre-harvest culture activities, can vary based on a wide range of factors, demonstrating a clear need for site specific studies (Schillaci et al. 2020; Jones et al. 2019).

Vibrio parahaemolyticus is naturally ubiquitous in the marine and estuarine environment, but only a small percentage of strains are recovered from clinical samples. A variety of genes are amplified by PCR to monitor for *V. parahaemolyticus*. Thermolabile hemolysin (*tlh*) is a *V. parahaemolyticus* species-specific gene (McCarthy et al. 1999; Taniguchi et al. 1986) that is widely used to detect this species especially because it is present in both pathogenic and non-pathogenic strains. Monitoring shellfish harvest areas for total *V. parahaemolyticus*, however, is not always useful for public health risk assessment. Several genetic markers have been useful as indicators of pathogenic strains, especially thermostable direct hemolysin (*tdh*) and thermostable direct hemolysin-related hemolysin (*trh*). Nearly all clinical isolates harbored *tdh* during the late 1980's, and *trh* was first discovered in 1987 from an isolate that did not contain the *tdh* gene (reviewed in Nilsson and Turner 2016). However, more extensive, recent work has also documented the *tdh* and *trh* genes in environmental, non-pathogenic *V. parahaemolyticus* strains (reviewed in Klein et al. 2014) and their absence in clinical isolates (Xu et al., 2015). The *tdh* and *trh* genes are still widely used to monitor pathogenicity of *V. parahaemolyticus*, as pathogenic strains typically have at least one of the genes (*tdh* or *trh*) (Honda et al. 1992; Kishishita et al. 1992; Park et al. 2004; Panicker et al. 2004; Nishibuchi and Kaper 1995; Shirai et al. 1990). In fact, pathogenic *V. parahaemolyticus* strains are increasingly associated with the presence of both of the *tdh* and *trh* genes (Banerjee et al. 2014; DePaola et al. 2003; Haendiges et al. 2015; Jones et al. 2012; Turner et al. 2013; Xu et al. 2015).

Only recently have detection methods been developed that allow for detection of actual pathogenic strains of *V. parahaemolyticus* in the Northeast US (Xu et al. 2015; Whistler et al. 2015). Variations of the *tdh* gene have been useful for this purpose: the *tdh*3 and *tdh*6 variations are common in clinically prevalent New England strains, while the *tdh*5 variation is commonly found in environmental *V. parahaemolyticus* strains that do not frequently cause infections (Xu et al. 2015). These new markers can be used for augmenting the traditional *tdh* indicator to

provide information on the presence of common clinically prevalent types of strains and to relate detection of these new markers to total (*tlh*+) and potentially pathogenic (*tdh/trh*+) *V. parahaemolyticus*. The application of this approach required field-testing to determine prevalence and sampling procedures required to enable tracking these pathogenic strains.

The following sections of this report focus on the Northeast states (New Hampshire, Massachusetts, Connecticut) where study sites were located, by providing more detailed background information, descriptions of study designs and presentation of results. The final section also frames these findings into regional and national contexts.

The goal of this study was to determine the dynamics of actual *V. parahaemolyticus* pathogens, along with total *V. parahaemolyticus*, as a result of different culture and harvest practices (at the three state study sites) that tend to increase *V. parahaemolyticus* levels and the controls intended to eliminate the impacts of these practices, while also steps taken to ensure that the risk of *V. parahaemolyticus* -borne illnesses are prevented are balanced with minimizing unnecessary time, equipment and labor burdens on oyster farmers. The objectives of this study were to:

-Evaluate the effectiveness of the key harvest and handling practices by showing significant reduction or elimination of actual risk (pathogenic *V. parahaemolyticus*).

-Monitor environmental and climate conditions that affect the distribution, occurrence and prevalence pathogenic *V. parahaemolyticus* in growing areas.

-Update risk assessments for *V. parahaemolyticus* that reflect local and sub-regional differences in environmental conditions and disease incidence.

-Determine risk indicators for *V. parahaemolyticus* in oysters in the Northeast US.

EXISTING REGIONAL DATA AND STUDIES

The first multistate outbreak of *V. parahaemolyticus* in the U.S. in almost a decade occurred in 2012 and continued into 2013, to a large part due to the introduction of a pathogenic Pacific Northwest strain (O4:K12) into the New England region (e.g. Martinez-Urtaza et al. 2013). In response, New England states and New York initiated Vibrio monitoring programs and began instituting pre- and post-harvest controls to prevent further illnesses.

V. parahaemolyticus **Monitoring and Local Environmental Conditions**

Current *V. parahaemolyticus* management and control measures instituted at the state and national levels depend heavily on water temperature data, while other climatic and environmental data can also inform characterization of *V. parahaemolyticus* risk conditions, at both local and regional scales (Urquhart et al. 2016; Hartwick et al. 2019). Conditions can change within years and can vary widely between years (Hartwick et al. 2019; Xu et al. 2015; Taylor et al. 2018), so monitoring at adequate spatial and temporal scales is necessary to inform management actions. The data collected during this project over time for untreated (control) oysters is a source of valuable information on the dynamics of background levels for total and pathogenic *V. parahaemolyticus*, allowing better determination of when *V. parahaemolyticus*

controls are most critical, thereby potentially relieving growers from unnecessary control activities during the shoulder (before or after highest risk) seasons.

To track environmental conditions, all three states use sources of water condition data that are close to critical growing areas. There are also regional monitoring systems, like NERACOOS and MARACOOS covering the Gulf of Maine and Long Island Sound, that provide information across the full Northeast region and at sites in the Northeast that have proven invaluable for interpreting *V. parahaemolyticus* dynamics (Xu et al. 2015). At the federal level, NOAA effort provides Vibrio Predictive Models for many regions, including the Northeast where models are available for Long Island Sound, Massachusetts and New Hampshire (Great Bay Estuary). These basic models use predicted surface air temperature data to estimate *V. parahaemolyticus* doubling times, and thus an early warning system of potential public health risks and guidance for when use of pre-harvest/harvest cooling practices is optimal.
https://products.coastalscience.noaa.gov/vibrioforecast/northeast/default.aspx#LI

NEW HAMPSHIRE: Vibrio monitoring in the NH Seacoast by UNH scientists began in the late 1960's (Bartley and Slanetz 1971) and has occurred periodically from 1989 to the present (O'Neill et al. 1990; Jones and Summer-Brason 1998). Since 2007, UNH scientists have tracked potentially pathogenic *V. parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae* in oysters, water, sediment and more recently in plankton at two sites in the Great Bay estuary, and in 2014 the NHDES began to conduct *V. parahaemolyticus* monitoring at Little Bay oyster farms in conjunction with laboratories at UNH and at NHDHHS. This program expanded to Hampton Harbor in 2019 with the establishment of two new farms there. Data from NHDES monitoring have been reported in annual Vibrio Risk Assessment reports (NHDES, 2019; Dejadon and Nash, 2020) and at the annual NH Aquaculture Meeting, while UNH monitoring data are reported in annual reports and have been published in several recent peer-reviewed papers. Urquhart et al. (2016) was based on 2007-2013 data, Taylor et al. (2018) was based on 2010-2013 data, and Hartwick et al. (2019) was based on 2007-16 data.

The Great Bay NERR and the Piscataqua Regional Estuaries Partnership (PREP) maintain datasondes located in close proximity to monitoring sites and the data they collect have been used to track water and shellfish conditions through 2019. 2014 was the first year that the NH Shellfish Program began to augment those data with HOBO water temperature and salinity meters placed at the exact study sites, and in 2018 -19 a new datasonde was added to the oyster farming area. In addition to the datasonde data, local climate data and other biological (plankton) and water condition and quality (nutrients) laboratory measurements on grab water samples were also useful for this project to explore as potential risk indicators.

Previous year monitoring suggests that summer monthly average air temperatures in the NH Seacoast have been increasing from at least 2016-18, with the majority of months being above previous historical averages (Dejadon and Nash, 2020; Hartwick et al. 2019). *V. parahaemolyticus* has been isolated at 1°C in New Hampshire oysters, although normally they are first detected in warmer water between late April and June (Jones et al. 2014). The timing for *V. parahaemolyticus* concentrations in oysters to reach peak concentrations since 2007 has occurred mostly during July and August prior to 2014, and they are no longer detected starting between October to early December. Since 2014, the NHDES monitoring has shown the highest

V. parahaemolyticus concentrations are now occurring in August and September (Dejadon and Nash, 2020). Several recent papers have reported how environmental conditions affect *V. parahaemolyticus* concentrations in oysters in New Hampshire. Urquhardt et al. (2016) showed that inclusion of chlorophyll *a* concentration to an empirical model otherwise employing only temperature and salinity variables offered improved the estimation capability for modeling the likelihood (presence) of *V. parahaemolyticus* in the Great Bay Estuary. Hartwick et al. (2019) found that the optimal predictive model contained water temperature and pH as environmental variables, along with photoperiod and the calendar day of study as other significant variables. The predictive model enabled relatively accurate seasonality-based prediction of *V. parahaemolyticus* concentrations for 2014-2016 based on the 2007-2013 dataset and captured the increasing trend in extreme values of *V. parahaemolyticus* concentrations. Thus, water temperature remains the dominant environmental variable for explaining variability in *V. parahaemolyticus* concentrations in New Hampshire, with other conditions contributing to variability.

MASSACHUSETTS: Since 2013, the State of Massachusetts Division of Marine Fisheries (MA DMF) has collected oyster samples from shellfish harvest areas around Cape Cod Bay, southern Cape Cod, the southern Massachusetts coastline, and Martha's Vineyard. Samples have been transported to both MA DMF and UNH laboratories for *V. parahaemolyticus* analyses. Environmental and water conditions have been measured using datasondes and probes, either by the MA Shellfish Program, or the Cape Cod Cooperative Extension Program, to record conductivity, total dissolved solids, depth, pH, dissolved oxygen, chlorophyll *a*, and turbidity data at 15 min intervals for this project.

In previous studies, trends for water temperatures when *V. parahaemolyticus* was first detected and when *V. parahaemolyticus* was most prevalent varied between sites (Schillaci et al. 2020). Water temperatures when *V. parahaemolyticus* was first detected (~13 °C) and when *V. parahaemolyticus* was most prevalent (~19 °C) were similar between both Cape Cod Bay sample sites (Duxbury Bay and Barnstable Harbor), whereas water temperatures in Katama Bay when *V. parahaemolyticus* was first detected (~17°C) and most prevalent (~24°C), were higher. Total and pathogenic *V. parahaemolyticus* concentrations significantly correlated with average water temperature for pooled statewide data, and all sites, with limited exceptions. Relationships between other environmental parameters and total and pathogenic *V. parahaemolyticus* abundance varied considerably between sites. Further, during periods of increased surveillance sampling, variability was observed in the background abundance of total and pathogenic *V. parahaemolyticus* at a spatial and temporal scale that the routine surveillance program would fail to capture, likely requiring a sampling frequency and sample size to that is not practical for most State programs. The monitoring for ST36 proved to be interesting but not that informative, as the detection of pathogenic strains (ST36 and 631) was rare and required an amount of work typical for research but beyond sustainable routine efforts, especially having to rely on live *V. parahaemolyticus* cultures of environmental isolates.

CONNECTICUT: Starting in 2014, the Connecticut Department of Agriculture Bureau of Aquaculture (DA/BA) has collected samples all along the CT coastline, accompanied by measurements of air and water temperature, salinity, and depth. Previous studies have found that the most significant predictive variable for total *V. parahaemolyticus* is bottom seawater temperature at the time of collection. In general, findings suggest that environmental total *V.*

parahaemolyticus is identified at low levels (<2.0 MPN/g) early in the Vibrio season when near-bottom and near-surface water temperatures are less than 20°C, and levels climb steadily through the summer as water temperatures increase. Total *V. parahaemolyticus* in the environment peaks when water temperatures are at their highest; during 2014 and 2015 near-bottom seawater temperatures reached 24°C to 25°C by the end of August and into early September (DeRosia-Bannick et al. 2016). During 2014, levels remained relatively elevated even as water temperatures dropped off through September, a phenomenon that also occurs in NH (Hartwick et al. 2019). In 2015, total *V. parahaemolyticus* dropped off rapidly as water temperatures dropped through September. A total of 101 shellfish samples were collected during 2014 and 2015 and *V. parahaemolyticus* was detected in 100 of the 101 samples collected during the study period. Median *V. parahaemolyticus* levels were 1.38 log MPN/g and ranged from the limit of detection (LOD =-0.52 log MPN/g) to 4.36. *V. parahaemolyticus tdh*+ was identified in 19 of 101 samples analyzed with median *tdh*+ levels of -0.44 log MPN/g, ranging from the LOD to 0.36 log MPN/g. *V. parahaemolyticus trh*+ was identified in 18 of 101 samples with median *trh*+ levels of -0.44 log MPN/g, ranging from the LOD to 0.36 log MPN/g. Finally, near-bottom temperature accounts for 18.7% of the variation in total *V. parahaemolyticus* when a simple linear regression model was applied. Oysters were the only implicated product from the 2013 outbreak, and past work from Long Island Sound has shown that oysters contain significantly higher levels of *V. parahaemolyticus* than clams and that *V. parahaemolyticus* levels in oysters had an inverse correlation with salinity (Jones et al. 2014).

Vibriosis Incidence in Each State

The FDA COVIS annual summary reporting of vibriosis and other illnesses in the US ceased to be published in 2014, so this consistent source of information has not been available in recent years to provide a single source and of directly comparative data for state illness rates. During and after the early shellfish-borne *V. parahaemolyticus* outbreaks in 2012-13, states in the Northeast began to hone their approaches for tracing illnesses to shellfish harvest areas as a part of newly required *V. parahaemolyticus* Control Plans. For shellfish harvesting and consumption, it is critical to distinguish *V. parahaemolyticus* cases from those caused by other *Vibrio* species, whether cases are shellfish-borne or otherwise, from what harvest area the implicated shellfish originated, and the date when the harvesting occurred.

NEW HAMPSHIRE: The incidence of shellfish-borne *V. parahaemolyticus* illnesses in NH has been published in NSSP-required annual *V. parahaemolyticus* risk evaluation reports since 2014. Prior to that, data for the overall incidence of vibriosis from all causes was available through the CDC in annual national reports. There has never been a *V. parahaemolyticus* outbreak in NH due to shellfish consumption or otherwise, and the number of annual vibriosis cases over the past decade has been between 4 and 16 (NHDPHS 2020). Recent *V. parahaemolyticus* cases associated with shellfish consumption include no *V. parahaemolyticus* cases linked by epidemiology and traceback information to oysters commercially harvested in New Hampshire waters in 2018 and 2019, and 1 case in 2017 (NHDES 2019; Dejadon and Nash, 2020).

MASSACHUSETTS: The incidence of shellfish-borne *V. parahaemolyticus* illnesses in MA has been reported by the Massachusetts Department of Public Health in annual *V. parahaemolyticus* risk assessment reports since 2012 as well as through CDC COVIS reporting. Prior to that, overall incidence of vibriosis from all causes was reported through the CDC in annual national

reports. Statewide sole source *V. parahaemolyticus* case occurrence increased from 2 in 2011 to 9, 33, 24, and 29 cases from 2012 to 2015, then decreased to 10 cases in 2016 (Schillaci et al. 2020). A study of 44 cases that occurred in MA during 2014-15 showed 75% of cases were attributed to Katama Bay and Western Cape Cod Bay, and most of those were attributed to *V. parahaemolyticus* strains 36 and 631. In addition, 42 of the 44 studied cases occurred from July 1 and September 15, with 85% of cases occurring when air temperatures were >24°C, though the actual risk per serving and timing differed between the two elevated-risk areas. Based on these trends, Massachusetts promulgated enhanced controls in the two regions where the majority of illnesses were reported (WCCB, Katama Bay), and for the period of peak illness occurrence (July 1 and September 15); highlighting how this type of analysis can be used to refine and complement general risk assessment methodology.

CONNECTICUT: The incidence of shellfish-borne *V. parahaemolyticus* illnesses in CT has been tracked using the current methodology since 2009 and documented in annual *V. parahaemolyticus* risk assessment reports. Prior to that, overall incidence of vibriosis from all causes was reported through the CDC in annual national reports. Confirmed *V. parahaemolyticus* cases linked to Connecticut/multistate (incl. CT) sources increased from 1/ 1-3 per year in 2010-12, to 23/11 in 2013, then down to 1/2 and 2/8 in 2014 and 2015 (DeRosia-Banick et al. 2016). There was a *V. parahaemolyticus* outbreak from oysters harvested in the Norwalk, Westport, Darien area in 2013 that included 23 confirmed CT cases.

Husbandry Practices and Research Focus in Each State

NEW HAMPSHIRE: Although some floating gear is permitted on a few farms, most oyster farmers use bottom culture, principally with cages. Some direct bottom planting occurs on some farms. Most oyster farmers use cages though a few use bottom planting. For this project, the NH Shellfish Program Manager recommended continuing with research to determine the time required for re-submergence of caged oysters to reduce *V. parahaemolyticus* to background levels following air exposure during warm weather. Interviews with 11 farmers and some less formal discussions revealed that the longest period of time that farmers expose oysters during the summer is between two and three hours.

MASSACHUSETTS: Aquaculture operations utilize a variety of culture methods in Massachusetts including bottom and floating cage culture and direct bottom planting of single oysters. Because the waters of Massachusetts in the summer time are highly productive and highly saline, growers must take steps to manage the colonization of fouling organisms on culture equipment and individual oysters. A common method utilized in Massachusetts to de-foul oysters prior to marketing, is to exposure them to the air for a period sufficient to kill the fouling organisms, but not result in lethal or sub lethal impacts to the oysters. The exact period of exposure varied based on ambient air temperature, the level of fouling, and grower preference, but generally range from 24 to 48 hours. Currently Massachusetts growers are required to re-submerge oysters for 10-days prior to harvest, a decrease from the initial requirement of 14-days based on DMF studies. The goal of this study was to identify if the required re-submergence time could be reduced even further. The impetus to reducing the period of resubmergence required is to limit the extent of recolonization that nay occur during the resubmergence interval.

CONNECTICUT: The CT Department of Agriculture Bureau of Aquaculture (DABA) instituted a policy following the outbreak that all oysters harvested from the 2013 outbreak area (Norwalk, Westport and Darien) must be cooled to an internal temperature of 50°F within 1 hour of harvest when water temperatures are greater than 68°F. Oyster harvested from growing areas outside of the 2013 outbreak area must be placed under refrigeration or on ice with 5 hours of the start of harvest, and must be cooled to an internal temperature of 50°F within 5 hours. The majority of oysters harvested during the summer months in Connecticut are rapidly cooled to an internal temperature of 50°F within 1 hour of harvest

Similarities among the three states:

- Reduce the internal temperature of shellfish to 50 °F or below as soon as possible during the state's warm *V. parahaemolyticus* risk season.
- Oysters shall be iced or placed under temp control for adequate cooling to ensure safety and quality of product.
- The plan applies to all oysters harvested for human consumption, not including seed oysters and recreational harvest. Connecticut offers rapid cooling guidance to recreational harvesters via the municipality responsible for issuing permits.
- Shellfish shall be shaded from direct sunlight while onboard vessels and during transport from harvest area to the original dealer.
- Each harvester shall keep a harvest logbook containing harvest area, date, start time of harvest, time to dock, amount harvested, time sold and time to refrigerate.
- Shellfish may not be placed in containers that do not have adequate drainage.

Differences:

Time to Temperature

- CT: All oysters harvested from the 2013 outbreak area (Norwalk, Westport and Darien) must be cooled to an internal temperature of 50°F within 1 hour of harvest when water temperatures are 68°F or above. Oysters harvested from outside of the outbreak area are subject to controls that require oysters to be placed under temperature control or iced within five hours from the beginning of harvest, and cooled to an internal temperature of 50°F within 5 hours.
- NH: All oysters harvested between May 1st and September 30th shall be cooled to 50 degrees F internal temperature within 4 hours of being placed under temperature control.
- MA: From July 1st through September 15th, all commercially harvested oysters from designated growing areas shall be adequately iced within 2 hours of time of harvest or exposure.

Risk/ *V. parahaemolyticus* Control Plan period

- CT: June 1st through September 30th rapid cooling is required for oysters harvested fron the 2013 outbreak area when water temperatures are 68°F or greater.
  NH: Starting May 1st and continuing through September 30th
  MA: Mid-May through mid-October.

METHODS AND RESEARCH APPROACHES

The overall research plan was to evaluate the effectiveness of key harvest and handling practices at reducing the risk of pathogenic *V. parahaemolyticus* infection and to determine risk indicators for *V. parahaemolyticus* in oysters in the Northeast US. As part of and in addition to field trials, we also monitored environmental and climate conditions that may affect the distribution, occurrence and prevalence of pathogenic *V. parahaemolyticus* in growing areas. The location and timing aspects of the design of field experiments were based on previous studies and disease occurrence patterns to update risk assessments for *V. parahaemolyticus* that reflect local and sub-regional differences in environmental conditions and disease incidence. We also used newly developed methods for detection of pathogenic *V. parahaemolyticus* strains, in concert with standard detection methods for total *V. parahaemolyticus* to discern what conditions may differentially drive an increase in pathogenic *V. parahaemolyticus* incidence. This study also is the first to simultaneously determine whether or not total *V. parahaemolyticus*, *tdh/trh* or regionally-specific pathogenicity markers are useful for estimating *V. parahaemolyticus* risk. These detection analyses were run on all samples to allow for a regional comparison.

State-Specific Research Approaches

Each State Authority decided on what husbandry practices to assess. In NH, sorting oysters while they are out of the water and exposed to the air for 1-3 hours is a common practice that needed further evaluation. In MA, de-fouling of oysters out of the water and exposure to air for up to 48 h was a practice in need of further evaluation. In CT, the major control practice that has resulted in the decline of *V. parahaemolyticus* illnesses from harvested oysters is well-defined time to temperature control. Each of the evaluated practices involved different degrees and extents of temperature abuse in at least one treatment. Based on past studies in the three states and elsewhere, it has been well established that this abuse of oysters increases total *V. parahaemolyticus* and *trh, tdh* marker concentrations, thus providing conditions conducive to being able to confirm control effectiveness for reducing or eliminating actual *V. parahaemolyticus* risk in all trials. In addition, we further ensured the likelihood that risk conditions would be present by conducting trials during high risk periods, based on review of prior disease and *V. parahaemolyticus* incidence data related to harvest sites and dates. The basic approaches are summarized in Table 1 with more detailed descriptions in the text that follows.

NEW HAMPSHIRE: Re-submergence after 3-h air exposure

Day 0	2 control
Day 0: 3 hour	2 exposed
2 days	2 exposed & 2 control
4 days	2 exposed & 2 control
7 days	2 exposed & 2 control
10 days	2 exposed & 2 control

MASSACHUSETTS: Re-submergence after 48-h air exposure

Day -2	3 controls
Day 0: 48h exposure	3 controls & 3 exposed
4 days	3 controls & 3 exposed

7 days	3 controls & 3 exposed
11days	3 controls & 3 exposed

CONNECTICUT: Temperature abuse for different times and surveys across coast

1A&B	2 immediate slurry
2A&B	2 3-hours to 50°F
3A&B	2 5-hours to 50°F
4A&B	2 12-hours to 50°F
Survey	duplicates at 4 sites
Survey/temp abuse	single samples per treatment per site

Table 1. Number and timing of treatments in each state.

NEW HAMPSHIRE: Re-submergence studies following air exposure of subsurface cultured oysters. There are two areas in NH coastal waters where oyster aquaculture occurs, however, most (26 of 28) licensed sites are in the Little Bay portion of the Great Bay Estuary. We conducted a total of 3 re-submergence field trials at an oyster farm in Little Bay NH during the summer of 2019. Beyond the determination of how long air-exposed oysters need to be re-submerged before *V. parahaemolyticus* levels and risks are reduced to background levels, our focus included determining the impact of early in the day exposure under overcast conditions where air temperatures did not exceed water temperatures, thus addressing the 'disturbance' factor apart from the elevated temperature factor.

Samples of market-sized oysters purchased from the on-site farmer were pre-allocated to mesh bags. One set of samples was held in a single bag that remained submerged during the trial to serve as the reference/untreated control, while the remaining samples were placed in another bag to serve as abuse/treatment samples. Initial reference (pretreatment) samples were collected when the exposed bag was removed from the water and air-dried in shade, which occurred ~1.5 h before low tide in late morning (10:45-11:45) to expose oysters to midday heat. Post-treatment samples were removed just prior to bag re-submergence following the 3 h drying periods, and thereafter from the re-submerged bag at 2, 4, 7 and 10 days. We tracked water temperatures by using HOBO pendants in bags suspended with the two oyster treatments and also measured water temperature and salinity at time of sample collection.

MASSACHUSETTS: Re-submergence studies following air exposure of cultured oysters for *V. parahaemolyticus* reduction (worse-case scenario model). Duxbury Bay was chosen as the study site due in part to the historic occurrence of oyster consumption related *V. parahaemolyticus* infections from this area. The sample period was chosen to be when the majority of illnesses have been reported. Duxbury Bay is a saltwater estuary on Massachusetts's South Shore with tidal exchange to Cape Cod Bay. The Bay is located between Duxbury Beach on the east, Saquish Neck on the southeast, and the mainland on the west. It is about 3 miles long, with an average width of 2 miles. The bay consists of tidal flats, mostly bare at low water, through which are several narrow and crooked channels. Duxbury Bay has an average depth of 10 feet at high tide, with water depths at mean low water ranging from 2' to -2', and an average salinity of 28 ppt. Duxbury Bay is well mixed and water temperatures in the summer range from 18- 21°C. 49 acres of commercial oyster farms operate in Duxbury Bay.

MA DMF conducted a total of 2 re-submergence field trials during 2019 in August and September for a total of 48 samples. Oysters in diamond mesh grow-out bags were distributed equally between oyster cages. The cages containing treatment samples were removed from the water and placed on an oyster barge for 24-48 h of desiccation. After 24-48 h, post-treatments samples were removed and the cage returned to the water. Duplicate treatment and control samples were removed at standard intervals (0 h, 2 or 4 days, 7 days, and 11 days of re-submergence).

CONNECTICUT: The study was comprised of two major components: 1) evaluating total and potentially pathogenic *V. parahaemolyticus* concentrations in oysters in relation to handling time using a time-temperature study (cooled to 50°F at 0, 3, 5, and 12 hours) in Norwalk and Westport, CT, and 2) assessing the prevalence of *V. parahaemolyticus* targets throughout CT using environmental background (iced immediately) vs. abused (iced after 12 hours, ambient temperature) oysters.

Time to Temperature Study. Oyster harvesting areas in CT are tracked in maps generated by the CT Bureau of Aquaculture (https://portal.ct.gov/DOAG/Aquaculture1/Aquaculture/Shellfish-Area-Classifications--Maps). Oysters were collected from Westport lot 224 (41 04.665, 73 22.357) on 7/9/19 and a shellfish lease located within the Norwalk Islands 2013 outbreak area, Norwalk lot 43 (41 04.487, 73 23.129) on 7/24/19, 8/6/19, and 8/20/19. Each of these biweekly samples was divided into four groups of 24 oysters: 1) rapidly cooled immediately (time 0), 2) rapidly cooled within 3 hours, 3) rapidly cooled within 5 hours, and 4) placed on ice within 12 hours. The baseline (time 0) group was immediately rapidly cooled to an internal temperature of 50°F or less using ice slurry, the 3 hour group was exposed to 2.75 hours on deck (ambient air temperature) then rapidly cooled in ice slurry for 15 min, and the 5 hour group was exposed to 4.75 hours on deck then rapidly cooled in ice slurry for 15 min. After each sample reached an internal temperature of 50°F in the ice slurry, the oysters were removed and placed on ice. The 12-hour group was exposed to ambient air temperature for 12 hours prior to placement on ice (no ice slurry). The actual average total time to 50°F per group was 0.9, 2.86, 4.98, and 12.85 hours, respectively. Ambient temperature data loggers were used to monitor the air temperature throughout the study, and internal Smart Button data loggers were placed in the oysters to monitor the change in internal temperature.

Environmental Abuse Study. The goal of the environmental abuse study was to assess background pathogenic *V. parahaemolyticus* populations in biweekly samples of untreated oysters and the impact of 12-hour temperature abuse on oysters throughout the different important CT growing areas through the potential risk season (June-September). This information is being used to inform and refine management strategies based on our understanding of the environmental triggers of these total and more virulent strains.

Paired samples were collected at each location (immediately iced [background]) and exposed to ambient air temperature for 12 hours [abused]). Samples were collected from Greenwich Mayhew North (41 00.627, 73 35.481), Norwalk lot 43, Westport lot 598 (41 04.737, 73 21.304), Stratford lot 437 southwest (41 07.862, 73 06.685), and Groton lot 30 (41 18.797, 71 59.275). Initially, 30 oysters were collected from Norwalk, Westport, Stratford, and Groton on 6/25/19

and were directly iced, providing duplicate background samples. A single 12-oyster sample was collected from Greenwich on 7/9/19 and was immediately iced, providing a single background sample. Subsequently, paired background/abuse samples were collected. 24 oysters were collected from Norwalk, Westport, Stratford, and Groton on 7/16/19 and 7/30/19, providing 12 background and 12 abused per sample. However, none of the Stratford 7/30 oysters were iced; therefore, they were processed as duplicate abused samples. All of the abused samples collected on 7/30 were accidentally immediately iced for 4 hours and 20 minutes and then removed from ice and were subsequently exposed to ambient air for 11-16 hours. 24 oysters were collected from Greenwich, Norwalk, Stratford, and Groton on 9/10/19, providing 12 background and 12 abused per sample. The samples directly placed on ice actually reached 50°F in a median time of 20 minutes and an average of 45 minutes because a few samples took over 2 hours to reach 50°F. The abused samples actually reached 50°F by a median 15 hours and an average 15.5 hours. When the abused samples from 7/30 that were accidentally initially iced for over 4 hours were removed from the average timing calculation, the actual average time to 50°F was 14.2 hours. Ambient temperature data loggers were used to monitor the air temperature throughout the study, and internal smart button data loggers were placed in the oysters to monitor the change in internal temperature.

V. parahaemolyticus **Analyses**

Oyster samples from Connecticut and Massachusetts were transported on ice to UNH researchers, and those from NH were received at the Jackson Estuary Laboratory (UNH) dock prior to processing. During processing, oysters were cleaned, shucked, weighed and mixed in equal weight with alkaline peptone water (APW). The oysters were homogenized and serially diluted, and subsequently *V. parahaemolyticus* were enriched overnight at 37°C (Kaysner and DePaola, 2004). Lysates were prepared from enrichment tubes by boiling, and 2µl of the cleared lysate was used as a template for PCR amplification. Amplicons were detected in real-time by fluorescence emission during 45 cycles of PCR to quantify *V. parahaemolyticus*. Samples were evaluated against negative (water) and positive (DNA from ST36 or ST631 *V. parahaemolyticus*) controls. The real-time PCR assays included simultaneous amplification of an internal amplification control (IAC) to account for inhibition of amplification from contaminants including oyster tissue present in enrichments that could lead to a false negative result (Nordstrom et al. 2007). Reactions where fluorescence reached the threshold (Ct value) after 42 cycles were run on an agarose gel to determine whether late fluorescence detection was due to amplification (band presence) or amplicon independent probe degradation (no band). Out of over 2000 reactions, 32 had Ct values above 42 and were run on an agarose gel, 84.4% had target amplification (band presence).

Our tiered analysis usually first employs detection of the species-specific *tlh* gene to enumerating total *V. parahaemolyticus* (Nordstrom et al. 2007). However, since we were evaluating use of tdh3/6 target as a marker for potentially pathogenic *V. parahaemolyticus* while the MPN enrichment tubes were fresh, we first screened the most concentrated homogenate enrichments for the presence of *tdh3/6* marker. Five to 10 isolates were collected from fresh *tdh3/6* positive enrichment tubes using Vibrio CHROMAgar (CHROMagar, Paris, France) and subsequently tested for ST36 (*tlh, tdh, trh, cps*) and ST631 (*tlh, tdh, trh, end*) using PCR assays (Whistler et al. 2015). As controls, isolates were collected from 8/12/19 MA and 7/15/19 enrichment tubes that

tested negative and positive for *tdh*3/6 and were subsequently tested for *V. parahaemolyticus* markers *tdh, trh, prp* and *cps* (Nordstrom et al. 2007; Whistler et al. 2015). Results were as expected, all isolates collected from *tdh*3/6 negative tubes were negative for pathogenic island markers (*tdh, trh, prp, cps, end*) and isolates from *tdh*3/6 positive tubes harbored pathogenic island markers (*tdh, trh*).

After the initial screening, we determined the MPNs of total *V. parahaemolyticus* from the distribution of MPN tubes positive for the species-specific *tlh* gene (Nordstrom et al. 2007; Kaysner and DePaola, 2004), and then in a third tier, *V. parahaemolyticus*-positive lysates were subjected to MPN quantification of bacteria harboring one or both hemolysins (*tdh* and *trh*) using fluorescent probes and primers specific to these genes (Nordstrom et al. 2007). The lysate from MPN tubes that tested positive for *tlh, tdh, trh*, and *tdh* 3/6 were tested for *prp, flp* (ST36) and *end* (ST631) using real-time PCR. For all samples that were positive for *tdh* 3/6, one or more enrichment tubes that tested positive for *tdh*3/6 were used to collect 5 to 20 isolates to determine if *tdh* 3/6 positive strains were present. For any CT, MA and NH sample that tested positive for *tdh* 3/6 in an enrichment tube, another enrichment tube that was not positive was also used as a control to confirm the absence of isolates containing *tdh* 3/6. All isolates that were *tdh* positive were then tested for *trh* and *tdh* alleles *tdh* 1, *tdh* 3/6 and *tdh* 5. Finally, *tdh* 3/6 positive isolates were tested for *prp, cps* and *end*.

Data and Statistical Analyses

The statistics and data analysis addressed the following questions: 1.) Do *V. parahaemolyticus* levels in 'exposed' oysters increase above background levels? 2.) Does re-submergence of exposed oysters reduce elevated *V. parahaemolyticus* levels to background levels? 3.) If so, how long does this take? 4.) Do time to temperature control measures for post-harvest shellfish eliminate or significantly reduce *V. parahaemolyticus* risk in terms of total *V. parahaemolyticus* and pathogenic markers?

For the purposes of data normality, all MPN/g values for *V. parahaemolyticus* concentrations were log transformed for all MPN data, including for all *V. parahaemolyticus* gene targets (*tlh, tdh, trh*, pathogen-specific targets) prior to statistical analysis. Samples with *V. parahaemolyticus* genes below the limit of detection were recorded as the limit value (e.g. <0.3 was analyzed as 0.3). Statistical significance was based on an alpha level of 0.05. The geometric mean and standard deviation were calculated for all replicate samples. Geometric mean *V. parahaemolyticus* concentrations for replicate control or background oysters in re-submergence trials were compared to *V. parahaemolyticus* concentrations in treated oysters for significant differences with post-hoc student t-test analysis. Differences between trial days were determined using ANOVA analysis and a non-parametric Mann-Whitney-Wilcoxon test. For the time-to-temperature trials, significant difference between the post-harvest times were analyzed using Repeated Measures One-Way ANOVA with Tukey's HSD post-hoc test, and all pairwise multiple comparisons were conducted using a Bonferroni t-test. Student's T-tests were used to determine differences in *V. parahaemolyticus* marker concentrations for paired background and abused oysters in the CT survey study. In addition, a One-Way ANOVA and a post-hoc Tukey's HSD test was used to determine significant differences among concentrations of the different *V. parahaemolyticus* markers. The frequency of *V. parahaemolyticus* (all measures) detection was

determined based on the number of samples analyzed. We note differences across growing area, the three-state region, and time of year as a basis to inform differences in management actions.

STATISTICALLY SUPPORTED FINDINGS

Data and interpretations were provided by personnel Connecticut, Massachusetts and New Hampshire and pooled to report the effects of temperature abuse, re-submergence or time to icing on *V. parahaemolyticus* abundance.

New Hampshire

New Hampshire data were collected between 8 July 2019 and 29 August 2019. Three 10-day trials were conducted with duplicate sampling beginning with time zero control and a three-hour exposure on day zero. Subsequent duplicate exposed and control oysters were collected on day two, four, seven and ten. The average daily water temperature ranged from 20.1 to 22.5°C with a minimum of 16.9°C and a maximum of 26.0°C (Figure 1). Total *V. parahaemolyticus* (*tlh*) and potentially pathogenic markers (*trh, tdh*) were detected in all samples for all three trials.

The first trial began at 10:45 AM on 7/8/19 and ended on 7/18/19. The air temperature increased by 2.2°C over the initial 3-hour exposure time on Day 0. The average daily water temperature for Trial 1 ranged from 20.1 to 21.4°C with a minimum of 16.9°C and a maximum of 24.5°C. The geometric mean total *V. parahaemolyticus* concentrations in un-exposed control samples remained relatively steady between 2.4 to 4.7 $x10^3$ MPN/g (Fig. 2). The geometric mean total *V. parahaemolyticus* concentrations in exposed oysters increased to 7.1 $x10^4$ MPN/g after the 3-hour air exposure, decreased to 2.4 $x10^2$ MPN/g after two days and continued to increase and decrease thereafter (Fig. 2). Due to the wide ranges and high standard deviations for the duplicate samples for each time and treatment, there were no significant differences between total *V. parahaemolyticus* concentrations in control and exposed oysters, although Day 2 exposed was marginally higher ($p = 0.087$) compared to Day 0 control oysters.

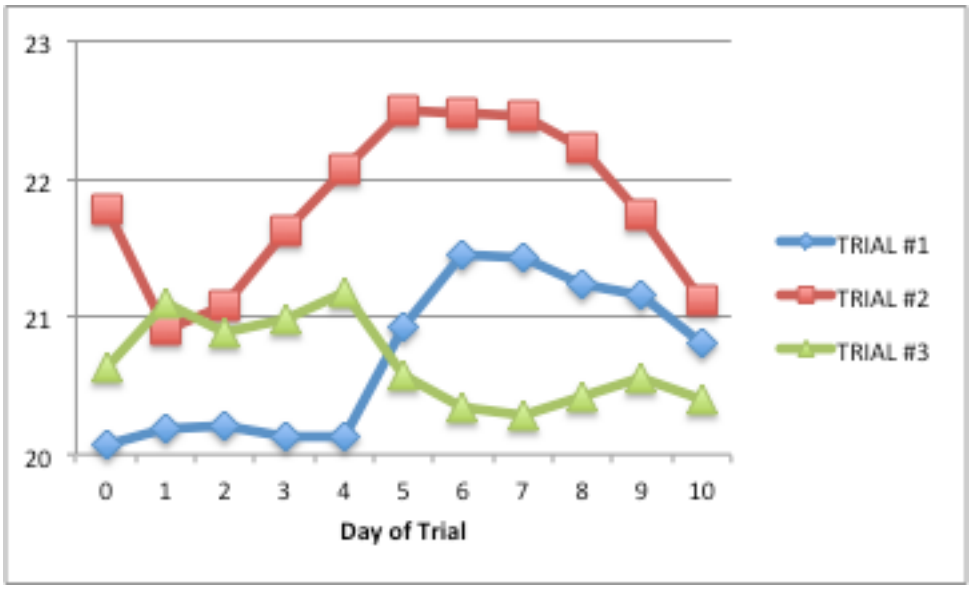


Figure 1. Average daily water temperature (°C) at the Little Bay study site for each of the three experimental trials

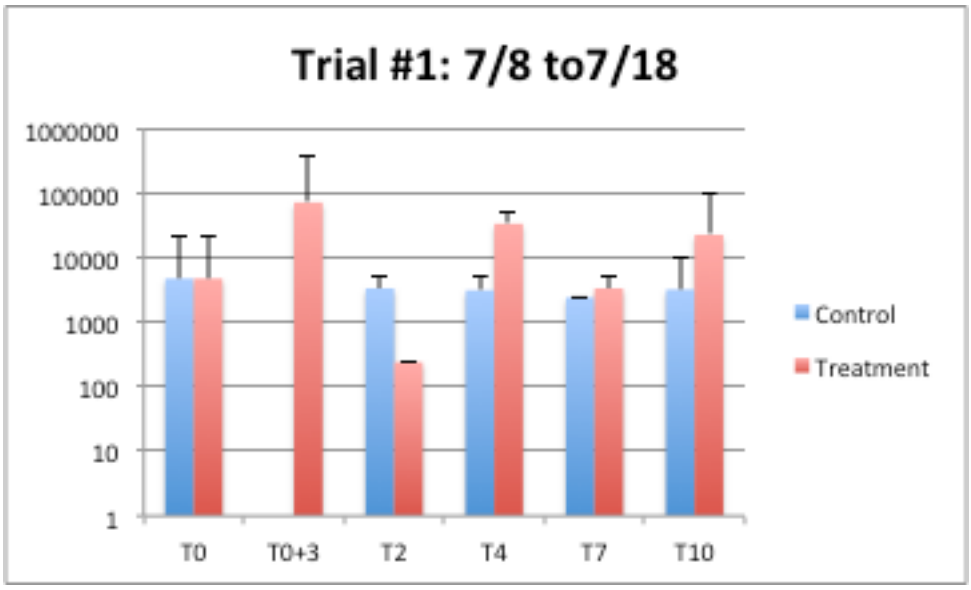


Figure 2. Geometric mean *V. parahaemolyticus* concentrations in air-exposed and control oysters during the 10-day Trial 1.

The second trial began at 10:50 AM on 7/22/19 and ended on 8/1/19. This trial was intended to show only the effect of disturbance of the oysters separate from exposure to elevated air temperatures. The day was heavily overcast with minimal wind. The air temperature was mild at 25.0°C and decreased by 0.6°C over the 3-hour exposure time on Day 0. The average daily water temperature ranged from 20.9 to 22.5°C with a minimum of 17.0°C and a maximum of 26.0°C. The geometric mean total *V. parahaemolyticus* concentrations in un-exposed control samples again remained relatively steady between 0.8 to 2.1 $x10^3$ MPN/g (Fig. 3). The geometric mean total *V. parahaemolyticus* concentrations in exposed oysters increased only from 1.2 to 3.3 $x10^3$ MPN/g after the 3-hour air exposure, remained relatively steady through 7 days then increased to 1.1 $x10^4$ MPN/g after ten days. Overall, total *V. parahaemolyticus* concentrations in exposed oysters were significantly higher than in control oysters, but there were no significant differences in concentrations by day.

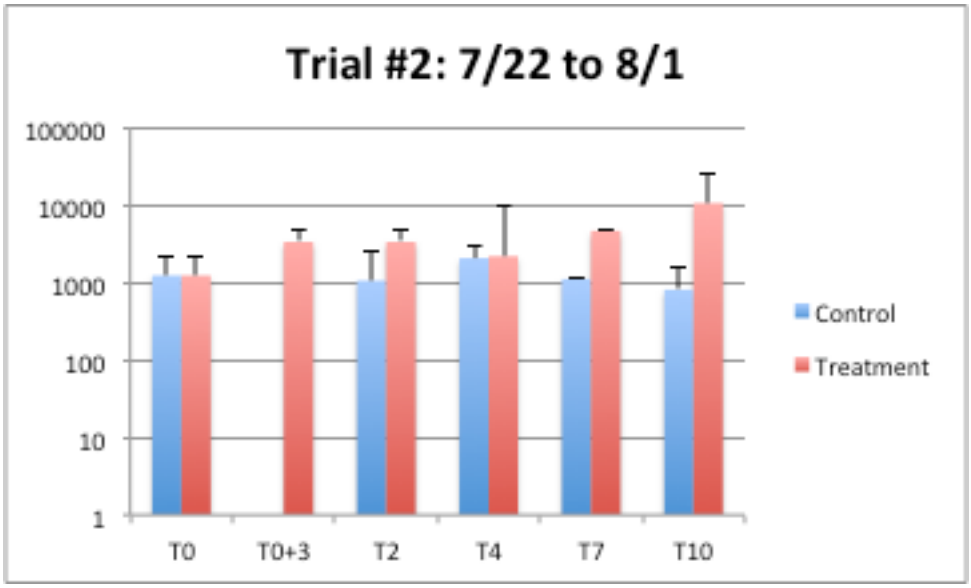


Figure 3. Geometric mean *V. parahaemolyticus* concentrations in air-exposed and control oysters during the 10-day Trial 2.

The third trial began at 11:45 AM on 8/19/19 and ended on 8/29/19. The air temperature increased by 2.8°C over the 3-hour exposure time on Day 0. The average daily water temperature ranged from 20.3 to 21.2°C with a minimum of 18.6°C and a maximum of 23.4°C. The geometric mean total *V. parahaemolyticus* concentrations in un-exposed control samples varied more widely than in the first two trials, ranging from 6.7 $x10^2$ MPN/g on Day 0 to 2.4 $x10^4$ MPN/g on Day 10 (Fig. 4). The geometric mean of total *V. parahaemolyticus* concentrations in exposed oysters increased from6.7 $x10^2$ MPN/g to 4.6 $x10^4$ MPN/g after the 3-hour air exposure, increased to 2.2 $x10^5$ MPN/g on Day 2 then both decreased and increased relative to controls thereafter. Overall *tlh* concentrations in exposed oysters were significantly higher than in control oysters and significantly different by Day. The comparison of Day *tlh* concentrations showed Day 2 was significantly higher than days 0, 4 and 7, and Day 7 was significantly lower than Day 10. Comparisons between days showed Days 3-hour (T0+3), 2, and 10 exposed and Day 10 control concentrations were significantly higher than Day 0 control, and Day 2 exposed was significantly higher than Day 7 exposed.

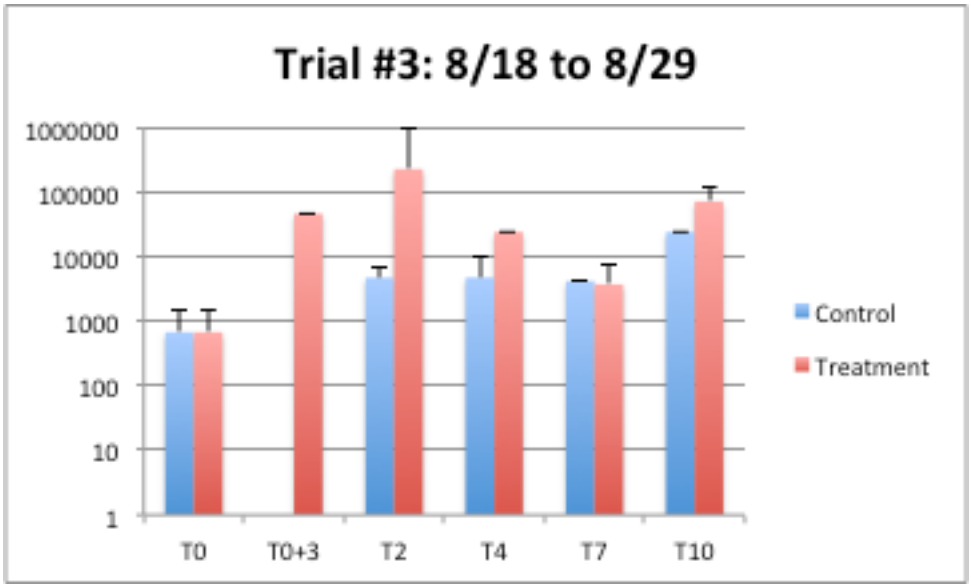


Figure 4. Geometric mean *V. parahaemolyticus* concentrations in air-exposed and control oysters during the 10-day Trial 3.

The effects of treatments on concentrations of *tdh* and *trh* in oysters were not consistent. Both *tdh* and *trh* concentrations in exposed oysters were higher than in control oysters for all time points in Trial 2, for most time points in Trial 3, but were inconsistent in Trial 1 (Fig. 5 A&B). The highest concentrations for both markers occurred on Day 2 and Day 10 in Trial 3.There were no significant differences between *trh* and *tdh V. parahaemolyticus* concentrations in control and exposed oysters in Trial 1, except the *trh* concentration in Day 4 exposed oysters was significantly higher than in Day 0 control oysters. There was also a significant difference in *V. parahaemolyticus trh* concentrations overall for Day/Treatment. Overall *tdh* and *trh* concentrations in Trial 2 exposed oysters were significantly higher than in control oysters, but there were no significant differences in concentrations by day. For Trial 3 *tdh*, the there was no overall difference between control *tdh* concentrations and exposed oyster *tdh* concentrations. Overall days were significantly different, with Day 2 concentration significantly higher than days 0, 4 and 7. For individual sample comparisons, the Day 2 exposed concentration was significantly higher than the Day 4 and 7 exposed concentrations, as well as higher than days 0 and 7 controls. Trial 3 *trh* concentrations were significantly different by Day and marginally ($p = 0.055$) by treatment, with Day 2 being significantly higher than Days 0 and 7.

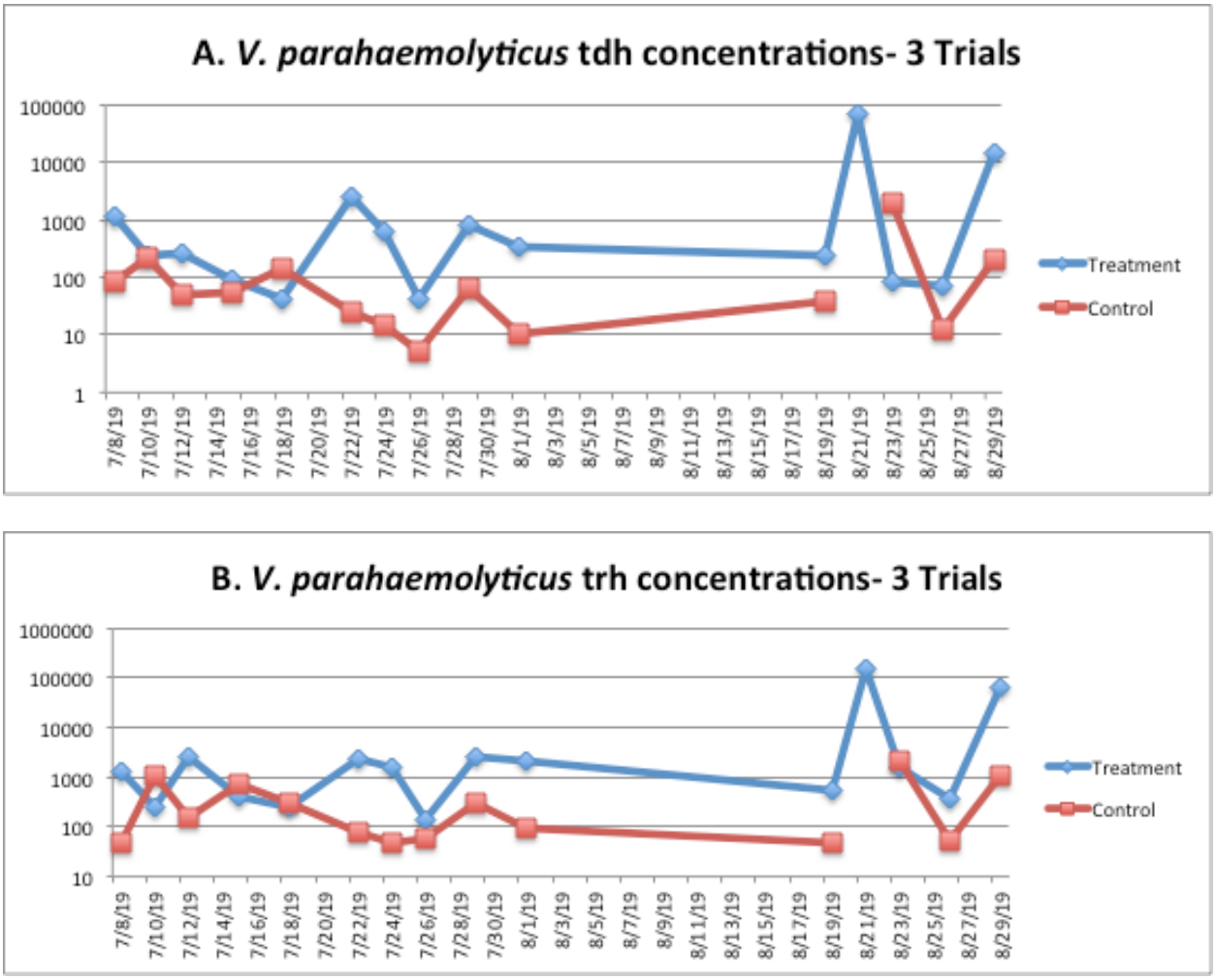
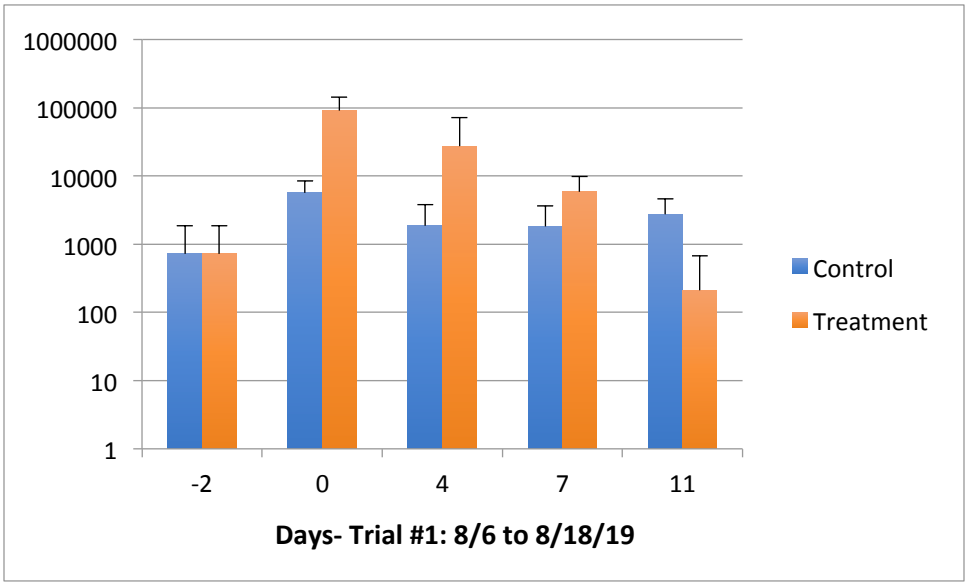


Figure 5. *V. parahaemlyticus* A. *tdh* and B. *trh* concentrations in Little Bay, NH oysters for all three trials.

Overall for all three trials, the geometric mean *V. parahaemolyticus* concentrations in exposed oysters were higher than in the controls in 10 out of 12 comparisons between Day 2 to Day 10, so they appeared to be elevated relative to the controls even though the differences were not always statistically significant. An ANOVA analysis of the exposed oysters showed that the exposed oysters did vary significantly by day ($p = 0.0189$). Day 2 was significantly different from Day 0 and Day 10 ($p = 0.032$) was significantly different than Day 2 ($p = 0.023$). This analysis highlights the apparent trend in the data where the geometric mean *V. parahaemolyticus* concentrations in exposed oysters increased on Day 10 compared to both control oysters and the Day 7 abused oysters. This was confirmed by a non-parametric Mann-Whitney-Wilcoxon test. Thus, the air abuse caused an increase in oyster *V. parahaemolyticus* concentrations though the differences between paired control and abused oysters were not significant. There was even less effect of disturbance without exposure to elevated air temperatures on treated oysters, although for all three trials the *V. parahaemolyticus* concentrations by Day 10 were consistently, though not significantly elevated compared to controls. Thus, Day 7 appears to be an acceptable time for re-submergence to be effective.

Massachusetts

Re-submergence trials in Duxbury Bay occurred in August and September 2019. The first trial was run from 8/6/19 to 8/19/19, and the air and water temperatures ranged from 22.6-24.2 °C and from 18.6 to 19.9 °C, respectively. The second trial began on 9/23/19 and ran until 10/1/19. The average daily air temperature declined steadily from 22.8 to 14.5 °C and the water temperature decreased from 21.0 to 16.5 °C from the beginning to the end of the trial. Background concentrations of *V. parahaemolyticus* were much lower during the September trial compared to the August trial (Fig. 6). The 24-48 h air exposure/abuse resulted in higher *V. parahaemolyticus* concentrations immediately following air exposure/abuse (Day 0) and on Days 2 or 4, though there were no significant differences between exposed and control oysters. By Day 7 after re-submergence concentrations were even more comparable and there were no significant differences in of *V. parahaemolyticus* concentrations in either trial.



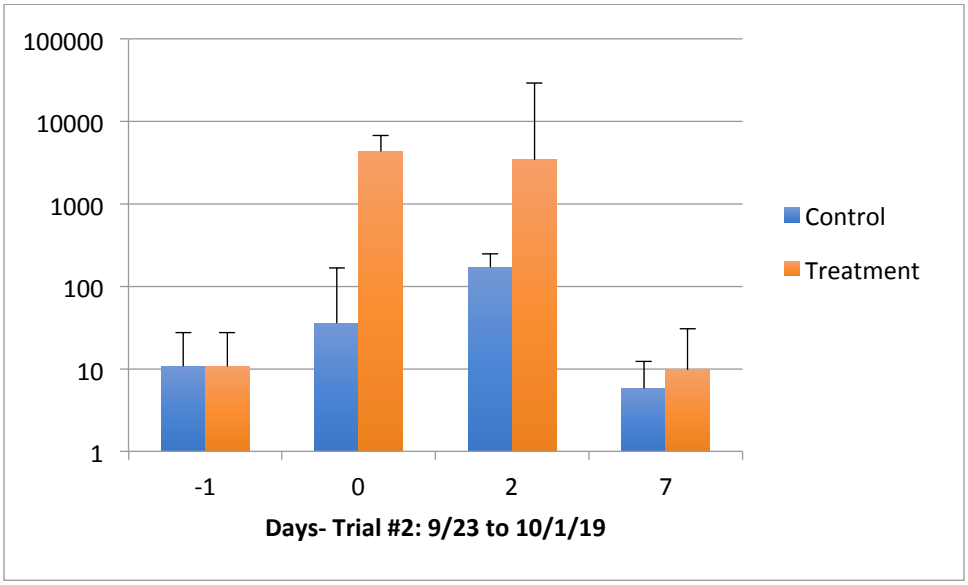
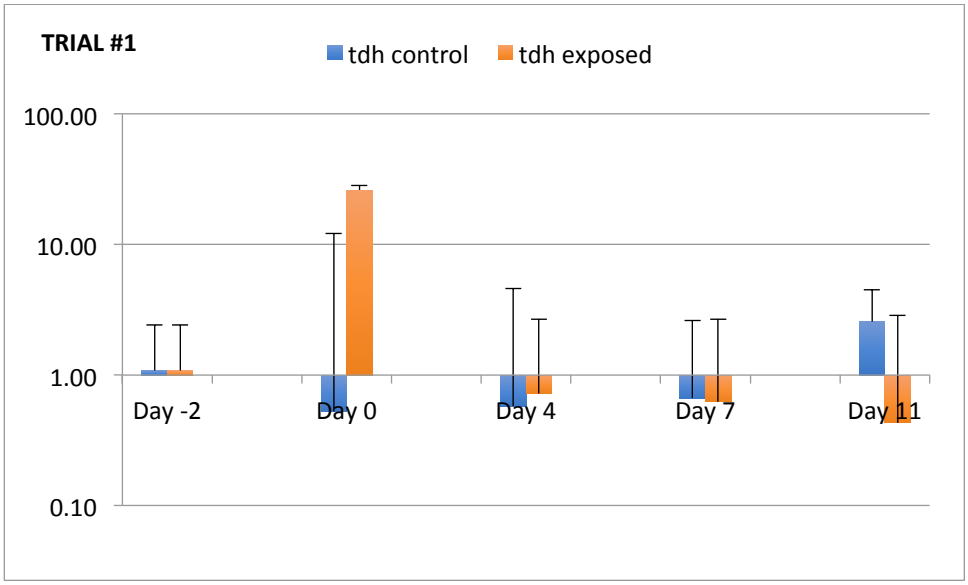


Figure 6. *V. parahaemolyticus* concentrations in abused and control oysters in Trials #1 (top) and #2 (bottom) in Duxbury Bay, MA.

Tdh was detected in 22 of the 27 samples in Trial 1 ((Fig. 7 top) and the highest concentration occurred after the 48 h air exposure. There was a significant difference in *V. parahaemolyticus trh* concentrations overall for Day/Treatment, however there were no significant differences between individual days or treatments. For Trial 2, *tdh* was only detected in one replicate Day 2 control sample. *Trh* was detected in all of the 27 samples in Trial 1 (Fig. 7 bottom) and there was a significant difference in *V. parahaemolyticus trh* concentrations overall for Day/Treatment and marginally ($p = 0.0596$) for Day. There were also significant differences between Day 0 exposed and both Day 0 control and Day 11 exposed oysters in Trial 1. For Trial 2, *trh* was detected in 11 of the 21 samples, with significant differences overall for Day and Day/Treatment, but only marginally ($p = 0.07$) for Treatment. Day 0 exposed oysters had significantly higher *trh* concentrations compared to Day 0 control, Day 2 control and exposed, and Day 7 control and exposed oysters. *Tdh* was only detected in one sample during Trial 2.



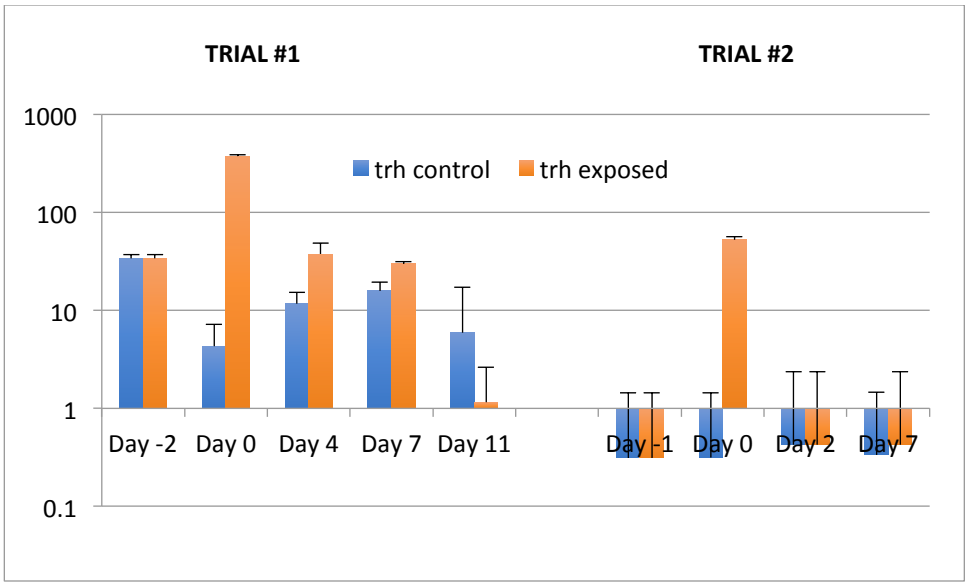


Figure 7. Concentrations of *V. parahaemolyticus* markers *tdh* (top) and *trh* (bottom) in abused and control oysters from two trials in Duxbury Bay, MA.

Overall, exposure of oysters to ambient air for 24-48 h in these two trials caused a significant temporary increase in *V. parahaemolyticus trh* concentrations that disappeared by the next time point. These results along with those for total *V. parahaemolyticus* and *tdh* concentration trends suggest that re-submergence for 7 days is sufficient to return *V. parahaemolyticus* concentrations in abused oysters back to background levels. These results are consistent with other studies conducted in Massachusetts.

Connecticut

TIME-TEMPERATURE STUDY: *tlh, tdh* and *trh* were detected in 100%, 87.5%, and 96.9%, of oyster samples in the temperature study, respectively. *tlh, tdh* and *trh*, were detected in 100%, 75% and 100%, of samples immediately iced (background), respectively.

While *tlh* can be elevated even in background samples (Fig. 8), the standard pathogenic markers, *trh* and *tdh*, were consistently low (under 100 MPN/g) until exposed to ambient air for 5 to 12 hours. There was a general upward trend for *tdh* and *trh*, and lesser so for *tlh*, the longer that oysters were exposed to ambient air (Fig. 8). There was a wide range of standard deviations, suggesting that there can be large variability even between paired *V. parahaemolyticus* samples (Figs. 9-12). Sometimes the duplicate samples had an order of magnitude difference, as demonstrated by Norwalk 8/6 at 0 hours (332,000±156,000 MPN/g), which had the highest *tlh* value and standard deviation during the study (Fig. 11). The elevated samples (>1,000 MPN/g) at time 0 were all *tlh* (Fig. 8): Norwalk 8/6 (332,000±156,000 MPN/g), 8/20 (2,940±5,920 MPN/g), and 7/24 (2,280±4,580); Westport 7/9 (1,280±7,670).

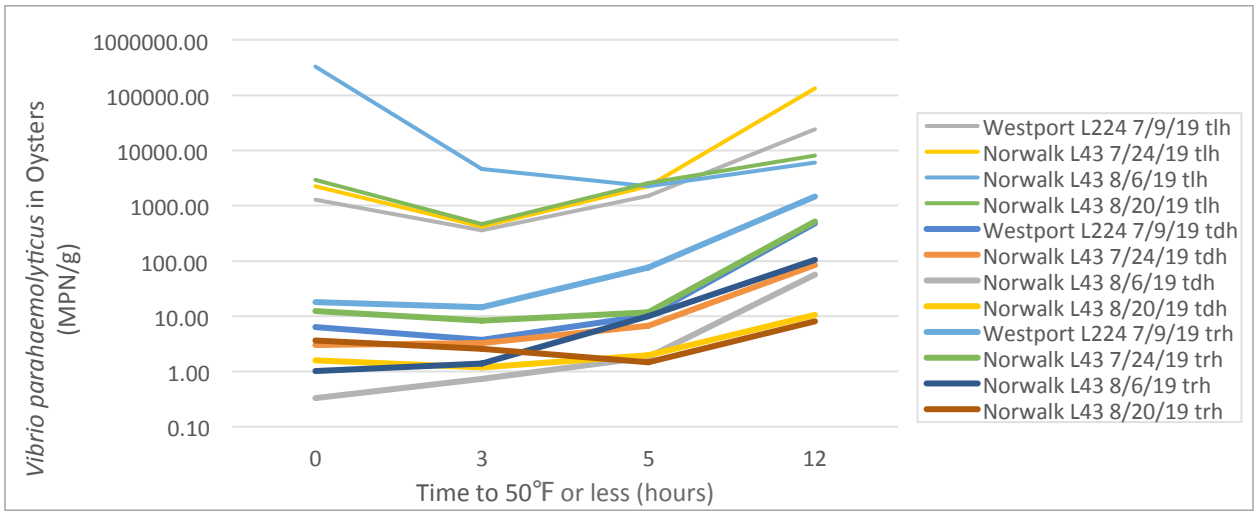


Figure 8. *Vibrio parahaemolyticus* marker concentrations in oysters over 12 hours post-harvest exposure to ambient air temperatures.

tlh concentrations were consistently greater than the pathogenic markers (Fig. 8), however, *trh* and *tdh* consistently increased after 5 hours post-harvest (Figs. 9-12). Comparatively, *tlh* had greater variability during the study and was higher at 12 hours post-harvest than all other times (0, 3, and 5 h post-harvest) in 3 trials (Figs. 9, 10, 12). *Tlh* was somewhat higher at 0 h in 3 trials and was lower at hours 3 and 5 (Figs. 9, 10, 12) and into hour 12 for Trial 3 (Fig. 11). Westport (7/9) had the highest *tdh* (474±955 MPN/g) and *trh* (1,450±2,930 MPN/g) levels recorded during the study (Fig. 9, hour 12), despite being the earliest sample of the year. For Norwalk, the highest *trh* (514±608 MPN/g) and *tdh* levels (83.5±12.8 MPN/g) were collected on 7/24 (Fig. 10).

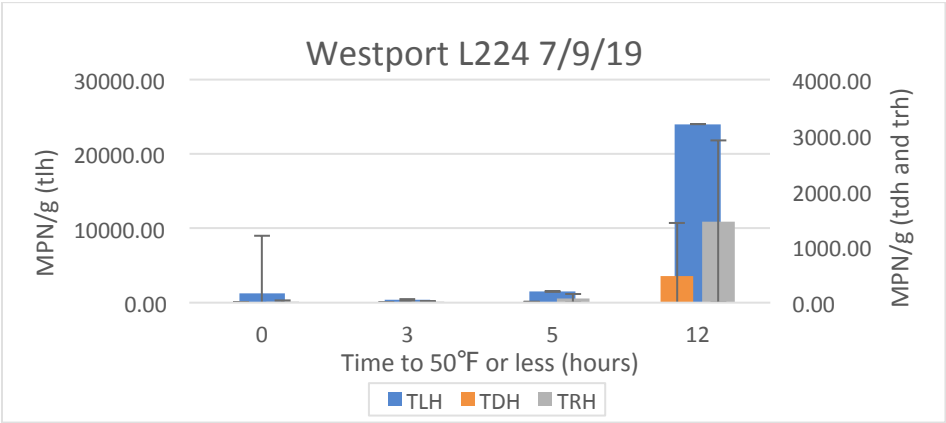


Figure 9. *V. parahaemolyticus* markers, *tlh* (blue), *tdh* (orange), and *trh* (grey), in oysters cooled to 50°F at 0, 3, 5, and 12 hours post-harvest for Westport lot 224 on 7/9/19-Trial 1.

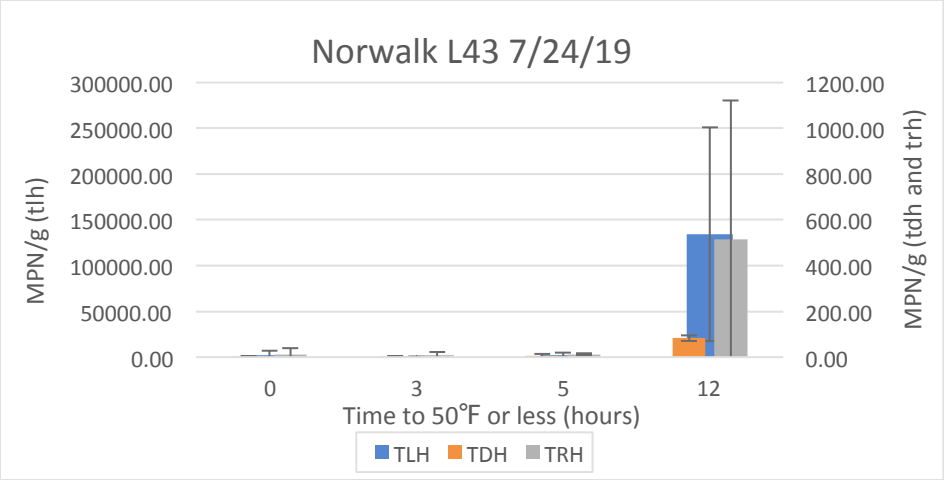


Figure 10. *V. parahaemolyticus* markers, *tlh* (blue), *tdh* (orange), and *trh* (grey), in oysters cooled to 50°F at 0, 3, 5, and 12 hours post-harvest for Norwalk lot 43 on 7/24/19-Trial 2.

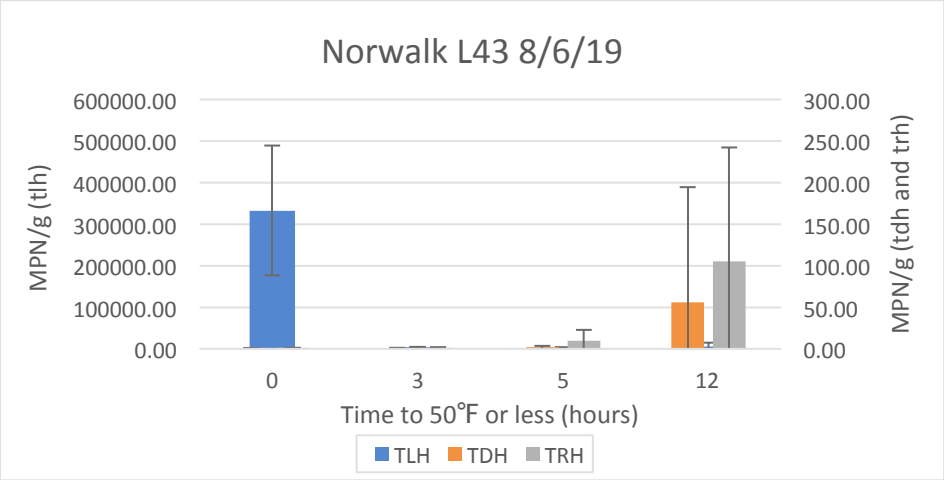


Figure 11. *V. parahaemolyticus* markers, *tlh* (blue), *tdh* (orange), and *trh* (grey), in oysters cooled to 50°F at 0, 3, 5, and 12 hours post-harvest for Norwalk lot 43 on 8/6/19- Trial 3.

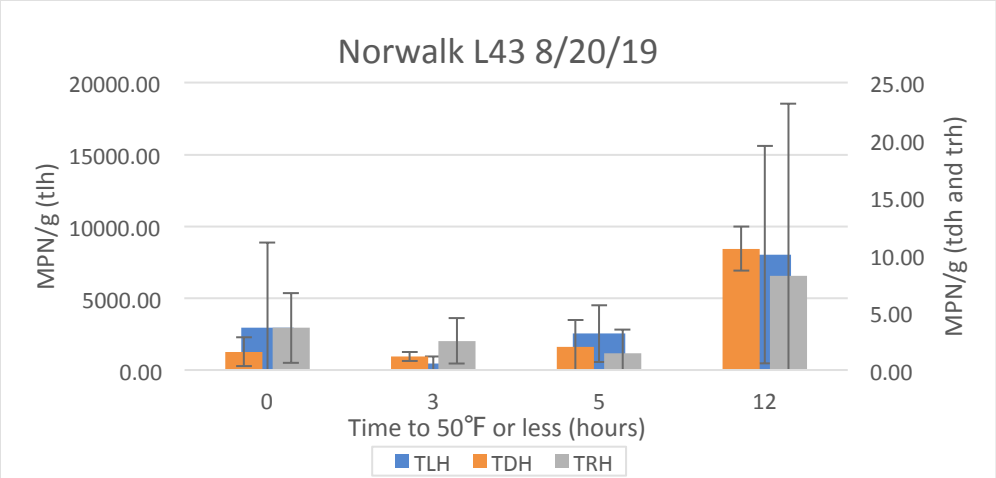


Figure 12. *V. parahaemolyticus* markers, *tlh* (blue), *tdh* (orange), and *trh* (grey), in oysters cooled to 50°F at 0, 3, 5, and 12 hours post-harvest for Norwalk lot 43 on 8/20/19-Trial 4.

Log transformed data were grouped based upon post-harvest time (e.g. 0, 3, 5, and 12 h), and analyzed using Repeated Measures One-Way ANOVA with Tukey's HSD post-hoc test. The Repeated Measures One-Way ANOVA was selected because four sample groups were collected from approximately the same location/date over time, and only a single factor was being compared (log MPN/g of each respective *V. parahaemolyticus* marker). The test determined that there was not a significant overall difference between the post-harvest times. Significant differences between *tlh* concentrations were seen in Trial 2 at the 3-h time point compared to twelve-hours ($p = 0.03$). Significant differences between hour zero and hour three, hour zero and hour five, and hour zero and hour twelve were observed in Trial 3 ($p < 0.01$).

Comparatively, the *tdh* and *trh* values showed significant differences among groups. For *tdh*, significant differences were found at a p value of <0.001 and an F value of 28.66. An all pairwise multiple comparisons (Bonferroni t-test) were completed and showed significant difference among the 0 and 12 h, 3 and 12 h, and 5 and 12 h post-harvest treatment groups ($p<0.001$). For *trh*, significant differences were found at a p-value of 0.001 and an F value of 13.04. An all pairwise multiple comparison (Bonferroni t-test) was completed and showed significant difference among the 0 and 12 h ($p = 0.003$), 3 and 12 hour ($p = 0.002$), and 5 and 12 h ($p = 0.014$) post-harvest treatment groups. Therefore, there is no significant difference between the *trh* and *tdh* levels between the 0, 3, and-5 h post-harvest treatment groups. This study established that in CT, harvesters could safely extend the rapid cooling time to allow up to 5 hours to cool shellfish to an internal temperature of 50°F. A more extended cooling may help to decrease shellfish mortality due to rapid cooling when water temperatures are most elevated.

Despite an increase in sea surface and bottom temperatures from June to August, the *V. parahaemolyticus* gene markers in oysters with the longest exposure time to ambient air temperatures did not track either of the temperature trends (Fig. 13).

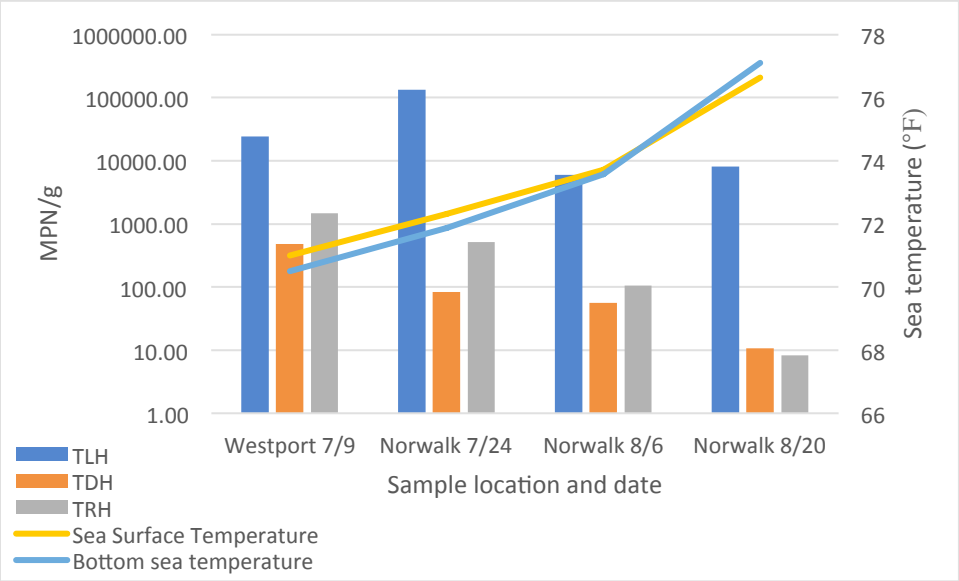


Figure 13. Concentrations of the *V. parahaemolyticus* markers, *tlh, tdh,* and *trh* in oysters after 12 hour abuse compared to water temperatures.

ENVIRONMENTAL ABUSE STUDY: *tlh, tdh* and *trh* detection was 85%, 45% and 55% in background (time 0) samples, while *tlh, tdh* and *trh*, were detected in 100%, 60.6% and 66.7%, of abused samples, respectively. The background samples had the highest readings during July. The highest marker *V. parahaemolyticus* concentrations were 2,400 *tlh* MPN/g (Greenwich 7/9, Westport and Groton 7/16), 9.2 *tdh* MPN/g (Norwalk and Groton 7/16), and 110 *trh* MPN/g (Stratford 7/16) (Fig. 14).

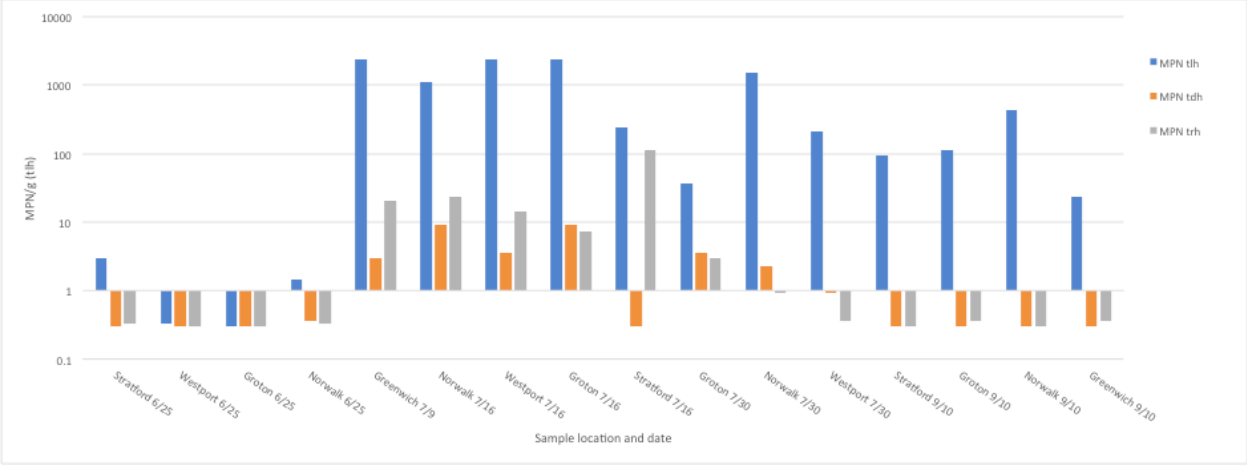


Figure 14. *V. parahaemolyticus* marker concentrations in oysters from background oyster samples immediately cooled to 50°F.

The abused samples had the highest readings on 7/30 (Fig. 15). The highest readings among the environmental abuse samples (12 hours to ice) all came from Stratford 7/30, with geometric

mean *tlh, tdh*, and *trh* levels of 1.45 ± 2.93 million, 10,390 ± 105,600, and 8,720 ± 25,500 MPG/g, respectively (Fig. 15). For Stratford 7/30, two abused samples were collected, but there was not a background sample. While both samples had high *V. parahaemolyticus* concentrations, one was more than an order of magnitude greater than the other. The higher sample had 4,600,000, 150,000, and 38,000 MPN/g for *tlh, tdh*, and *trh*, respectively. After Stratford 7/30, the highest *tlh, tdh*, and *trh* values in abused samples were 240,000 (Norwalk and Westport 7/16, Norwalk 7/30), 3,800 (Norwalk 7/30), and 4,600 MPN/g (Groton 7/16), respectively (Fig. 15).

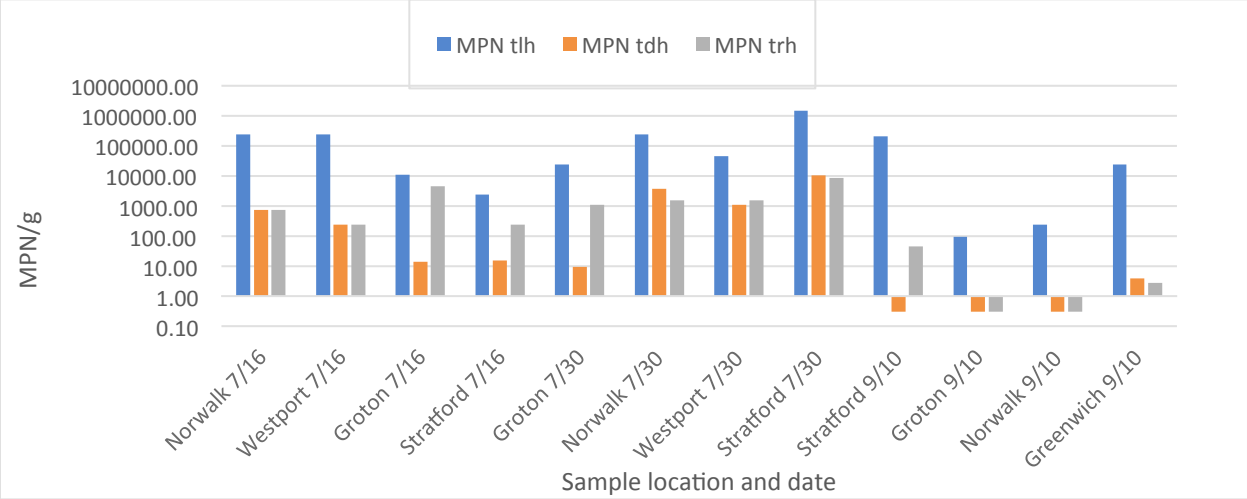


Figure 15. *Vibrio parahaemolyticus* marker concentrations in abused oyster samples.

Paired abuse samples had higher levels of *tlh, tdh*, and *trh*, relative to paired background samples (Fig. 16). The data were log transformed and standard t-tests were used to determine differences in *V. parahaemolyticus* marker concentrations. There was a significant difference between the background and abused sample groups ($p < 0.001$ for *tlh*; $p = 0.001$ for *trh*; $p = 0.007$ for *tdh*).

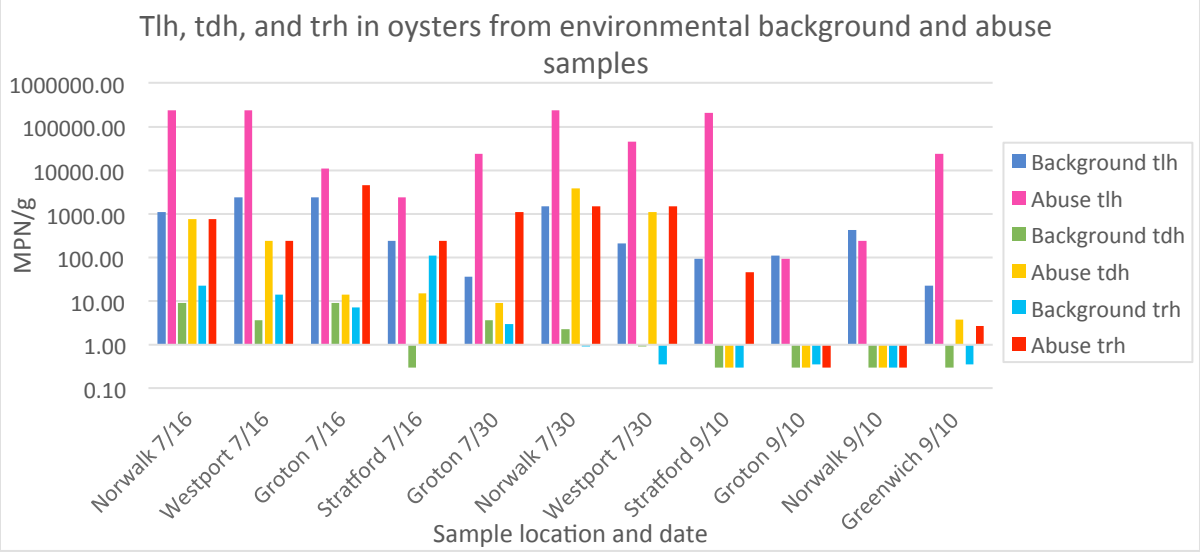


Figure 16. *V. parahaemolyticus* marker concentrations in background (dark blue-*tlh*, green-*tdh*, and light blue-*trh*) and abused (pink-*tlh*, yellow,-*tdh* and red-*trh*) oyster samples.

There was no significant difference for *tlh, trh*, or *tdh* levels in background (time 0) oysters or 12 hour abused oysters between towns. For background samples, p-values ranged from 0.559-0.946. For abused samples, p-values ranged from 0.313-0.456.

DIFFERENCES IN GENE PREVALENCE: *Tlh, tdh, trh*, and *tdh* 3/6 concentrations in background oyster samples from both studies were log transformed. A One-Way ANOVA showed there was a significant difference among the groups ($p < 0.001$). The ad hoc Tukey's HSD test identified significant difference between the *tlh* and *tdh* 3/6 and *tlh* and *tdh* values ($p < 0.001$), *tlh* and *trh* values ($p = 0.017$), and *trh* and *tdh* 3/6 values ($p = 0.003$). *Tdh* and t*dh* 3/6 ($p = 0.072$) and *trh* and *tdh* ($p = 0.723$) were not significantly different among background samples. Likewise, marker concentrations detected in abused (12-h ambient air exposure) samples from both studies were significantly different ($p<0.001$). The Tukey Test showed significant differences between *tlh* and *tdh* 3/6 ($p<0.001$), *tlh* and *tdh* ($p = 0.004$), and *trh* and t*dh* 3/6 ($p = 0.031$). While not significantly different in either analysis, the data suggests that *trh* may be more prevalent or consistently reach higher levels than *tdh* in CT because *tlh*, which reliably has the most elevated samples, was significantly different from *tdh* for both background and abused samples, but was not significantly different from *trh* in the abused samples. Additionally, *tdh* 3/6, which consistently had the lowest levels, was significantly different from *trh* but not *tdh*.

Characterization of *tdh* 3/6 Positive *V. parahaemolyticus* Isolates

All sample enrichment tubes positive for *tdh* 3/6 were used to collect 1 to 20 isolates to see if *tdh* 3/6 positive strains were present (Table 2). For all CT, MA and NH samples that tested positive for *tdh* 3/6, one enrichment tube that was not positive was also used as a control to confirm the absence of isolates containing *tdh* 3/6. Most of the samples were also tested for other indicator markers for ST36, including *prp, flp* and for ST631, *end*. In NH, 3 samples were positive for *tdh* 3/6 (MPN = 0.36/g) but none of these yielded *tdh* positive isolates (Table 2-C).

A. Summary of isolates collected from CT enrichment tubes.

Date & sample type	*tlh*	*tdh*	*trh*	*tdh3/6*	*prp*	*flp*	*end*	# of isolates[B] & marker test: *tdh,trh,prp,cps*
	MPN/g				Presence/absence in *tdh3/6* positive tube			
7/9/18 Westport 0-hour	11000	9.2	120	4.3	+	-	-	1/5 *tdh*6 (sequenced), *trh*

7/9/18[A] Westport 0-hour control tube tested neg tdh3/6	11000	9.2	120	4.3	+	-	-	1/5 *tdh5* (sequenced), *trh*
7/30/19 Stratford Exposed	$4.6x10^7$	150000	38000	1.1	+	+	-	*10 Negative*

B. Summary of isolates collected from MA enrichment tubes.

Date & sample type	*Tlh*	*tdh*	*trh*	*tdh3/6*	*prp*	*flp*	*end*	# of isolates[B] & marker test: *tdh,trh,prp,cps end*
	MPN/g				Presence/absence in *tdh3/6* positive tube			
8/6/19 Control	750	0.92	110	0.36	+	+	-	*10 Negative*
8/6/19 Control	210	0.92	24	0.36	+	-	+	*10 Negative*
8/8/19 Exposed	150000	2.1	24	1.1	+	+	-	1/15 *tdh, trh (tdh1-,tdh3/6-, tdh5-)*
8/8/19 Exposed	110000	30	930	2.9	+	+	-	1/25 *tdh* 2/25 *tdh*, *trh(all tdh1-, tdh3/6-, tdh5-)*
8/8/19 Exposed	46000	280	2400	0.72	+	+	-	1/10 *tdh, trh (tdh1-,tdh3/6-, tdh5-)*
8/12/19 Control	4600	1.1	24	0.3	+	-	-	1/20 *tdh5, trh*
8/12/19 Control	920	ND	2.8	0.3	+	+	-	1/10 *tdh, trh (tdh1-,tdh3/6-, tdh5-)*
8/12/19 Control tube tested neg tdh3/6	920	ND	2.8	0.3	-	+	-	*10 Negative*
8/15/19 Control	4300	1.1	3.6	0.3	+	+	+	*10 Negative*
8/15/19 Exposed	4300	0.74	24	0.3	-	+	-	*10 Negative*

8/15/19 Exposed	11000	1.1	46	0.74	+	+	+	1/20 *tdh3/6, trh*
8/19/19 Control	4300	4.3	24	0.36	+	+	-	5/10 *tdh3/6, trh, cps*

C. Summary of isolates collected from NH enrichment tubes.

Date & sample type	*Tlh*	*tdh*	*trh*	*tdh3/6*	*prp*	*flp*	*end*	# of isolates[B] & marker test: *tdh,trh,prp,cps*
	MPN/g				Presence/absence in *tdh3/6* positive tube			
7/15/19 Control Day 7 tube tested negative tdh3/6	2400	43	460	-				*10 Negative*
7/15/19 Exposed Day 7	4600	210	110	0.36	+	+	-	1/14 t*rh, prp* 2/14 *prp*
7/26/19 Control Day 1	1500	7.4	150	0.36	-	+	-	*10 Negative*
8/1/19 Exposed Day 10	4600	74	270	0.36	+	+	-	*1 Negative*

[A] Control enrichment tube tested negative for *tdh* 3/6
[B] Test isolates for *tdh* allele and *end,* and sequence any of interest.

Table 2. Concentrations of target *V. parahaemolyticus* markers in oyster samples and genotyping of *V. parahaemolyticus* strains from *tdh* 3/6 positive enrichment tubes from A.) Connecticut, B.) Massachusetts and C.) New Hampshire.

In CT, one of five isolates from the 7/9/19 Westport sample with an MPN of 4.3 *tdh* 3/6/g harbored *tdh* 6 (tube positive for *tdh* 3/6) or *tdh* 5 (control tube negative for *tdh* 3/6) (Table 2-A). The *tdh* 6 isolate was not ST36. None of the 10 isolates from the 7/30/19 Stratford sample (MPN = 1.1 *tdh* 3/6/g) were positive for any targeted markers. In MA, 13 samples from Trial 1 were positive for *tdh* 3/6 (MPNs = 0.3 to 2.9 *tdh* 3/6/g) and 8 of these 13 samples were also positive for *prp* and *flp* (Table 2-B). Twelve of these *tdh* 3/6 positive tubes were used to isolate *V. parahaemolyticus* strains. Six of the t*dh* 3/6, *prp* and *flp* positive samples yielded 1-2 isolates positive for *tdh*, and one other *tdh* 3/6 positive sample yielded one *tdh* positive isolate.

The frequency of detection of *tdh* 3/6 was low in New Hampshire and Connecticut (<5%) as well as in Massachusetts for the later season Trial 2 (0%), but relatively high (48%) in Massachusetts during Trial 1 (Table 2). The frequency of oyster samples containing *tdh* 3/6 was nearly equal for control and abused samples in all three states.

State	Number of samples	Samples with *tdh* 3/6 detection	Control/abused samples with *tdh* 3/6 detection
NH	58	3	½
MA-Trial #1*	27	13	6/7
MA-Trial #2*	21	0	0
CT	57	2	1/1

*MA Trial 1: 8/6/19 to 8/19/19. Trial 2: 9/23/19 to 10/1/19.

Table 3. Detection frequency of regionally-specific *V. parahaemolyticus* pathogenicity marker *tdh* 3/6.

RECOMMENDATIONS FOR IMPROVING RISK ASSESSMENTS IN SHELLFISH IN THE NORTHEAST

This study and our interpretation of results is structured in a way that focuses on providing critical background information and localized data needed inform managers of the best means of implementing the three major aspects of *V. parahaemolyticus* risk assessment and management:

1.) Management strategies, particularly time to temperature controls and re-submergence time requirements;
2.) Dynamics of the absolute and relative abundance of total *V. parahaemolyticus* and regionally-significant pathogenic strains implicated in clinical infections;
3.) Environmental conditions associated with increases in the absolute and relative abundance of total *V. parahaemolyticus* and regionally-significant pathogenic strains, and associated with the degree of risk.

This section of our report focuses on cross-regional differences and similarities that can help inform managers of the elements of *V. parahaemolyticus* risk assessment methodology and management that may be applicable at the regional level, versus those that need to be scaled to the state or harvest area- by- harvest area level.

There were study findings that showed some consistency between states. For example, in NH and MA, periods of air exposure associated with culture practices consistently resulted in an increase in oyster *V. parahaemolyticus* concentrations, though the difference between paired control and abused oysters were not always significant; with the extent of the increase probably depending on the initial *V. parahaemolyticus* levels and environmental conditions at the time of trials. Overall, when the difference between paired control and abused oysters were significant, 7-days of re-submergence was sufficient in both MA and NH to return *V. parahaemolyticus* concentrations to background levels.

However, generally speaking, we observed significant variability between states in a number of important areas with management implications. For example, in CT we did not observe a significant increase in *V. parahaemolyticus* concentrations in oysters that were abused for 5

hours, and in some cases after 12 hours, of ambient air exposure. These findings suggest that CT harvesters may safely extend the rapid cooling period from 1 hour to 5 hours from harvest to an internal temperature of 50°F. This is beneficial as a more gradual cooling period during periods when water temperatures are most elevated may help to decrease shellfish mortality associated with the physiological shock of rapid cooling.

Other observations include the minimal impact of only disturbance without elevated air temperature exposure in NH, although for all three NH trials concentrations in exposed oysters on Day 10 were consistently, though not significantly, elevated compared to controls and to Day 7 exposed concentrations. Both of these findings will be the subject of further study during the summer of 2020 as part of a related ongoing study in NH, MA and ME. The decrease in *V. parahaemolyticus* concentrations in Trial 4 in CT even after 12 h abuse illustrates the importance of conducting multiple trials to enable capturing worst case conditions as well as the potential for fine-tuning management strategies by time of year.

Dr. Whistler's group has established a framework to identify and track regionally specific pathogenic *V. parahaemolyticus* strains that can be transferred to other regions. Its application requires regional cooperation including sharing of critical clinical information, and is challenging because of the low incidence of specific pathogenic strains in nature, which, based on this study, is apparently the case at least for this region. Concentrations of the more traditional markers, *tdh* and *trh*, increased in response to over-exposure and abuse in MA and CT, though somewhat more inconsistently in NH. In addition, concentrations of these markers rapidly decrease following re-submergence. In contrast, the infrequently detected *tdh* 3/6 did not show a consistent trend relative to oyster exposure and re-submergence, but did disappear from later (September- in MA) study times. These finding suggest that there are different dynamics for total, *tdh/trh* and regional pathogenic strain concentrations in response to temperature abuse and exposure. Given the success of management strategies in the 3 states to illnesses by reducing exposure and temperature abuse of harvested oysters, the response observed in this study for traditional markers suggests these are useful indicators of increased risk related to oyster aquaculture husbandry practices.

States have set up monitoring programs to track environmental and potential risk conditions, and research and partner monitoring efforts in each state inform these programs. There are many environmental variables that may be important risk indicators, and a recent paper by Hartwick et al. (2019) builds on other previous studies in the Northeast to provide a framework for how to assess a wide range of different environmental risk indicators at a scale appropriate for harvest areas. Water temperature was again the most significant variable, but pH and other types of variables allowed for predicting peak total *V. parahaemolyticus* concentrations. This approach allows for prediction of peak timing for other variables, and thus alignment of variables to infer synoptic and pre-disposing conditions that may affect risk.

This study was not a monitoring study designed to determine how environmental conditions affect risk statewide, rather the focus was husbandry practices and air/water temperatures. As far as existing risk indicator evaluation is concerned, air and water temperatures are the overriding most important region-wide indicator in use at this time. This study began later than intended due to the very low *V. parahaemolyticus* levels prior to early July, so the onset of elevated *V.*

parahaemolyticus levels was later than expected based on water temperatures. The most comprehensive assessment of water temperature effects on *V. parahaemolyticus* concentrations was in CT, where despite increasing temperatures from July through August, the *V. parahaemolyticus* concentrations in abused oysters did not follow that trend. It appears that once a threshold temperature is reached and *V. parahaemolyticus* concentrations are relatively high in oysters, further changes in water temperatures did not track with *V. parahaemolyticus* concentrations. The study period extended over July and August in NH and CT, but was extended from mid-September into early October in MA. The results for the 2nd MA trial showed much lower total and *trh V. parahaemolyticus* concentrations, and the disappearance of detectable *tdh* and *tdh* 3/6, suggesting that risk indicators decrease in early fall with falling water temperatures, which is consistent with the timing of illnesses in MA (Schillaci et al. 2019). Further monitoring studies are needed to determine the potential for tracking markers of regionally significant pathogenic strains to be developed into a useful risk indicator.

One critical variable not yet effectively incorporated into environmental and husbandry aspects of risk assessment is the timing and locations of illness-linked shellfish. Schillaci et al. (2019) do consider this and have observed some interesting, though inconsistent, trends with environmental *V. parahaemolyticus* concentrations and water temperature. In New England, illness timing has been relatively consistent, but that might not always be the case with increasing water temperatures, and in regions where there may be more frequent early 'shoulder season' illnesses that do not fit gradual water/air temperature changes. This added complexity could be accommodated with the Hartwick et al. (2019) forecasting approach that can be adjusted using day of year.

TRANSFERABILITY TO OTHER SHELLFISH PRODUCING AREAS

Earlier the similarities and differences between states in their management of husbandry practices and *V. parahaemolyticus* control plans were described (Table 1). The findings of this study help to reinforce these plans and guidance, reflecting spatial and temporal differences and similarities, as well as ubiquitous differences in grower practices. The recent paper by Hartwick et al. (2019) is a useful framework for considering a wide variety of environmental risk indicators at the harvest area scale. Multiple applications of the approach where environmental data are available at the growing area scale are required for potential identification of significant environmental risk indicators in New England and any other region.

Beginning in 2014, Connecticut managers instituted rapid cooling controls and now have 5 years of data showing a significant reduction in illness as compared to 2013, prior to the implementation of rapid cooling controls. When considering the effective reduction in illness that has been achieved in Connecticut, the evidence is clear that rapid cooling to an internal temperature of 50°F within 1 hour of harvest is a successful management strategy for reducing the risk of illness associated with *Vibrio parahaemolyticus* (Figure 17). In 2019, although a number of background environmental samples and control samples showed extremely elevated levels of *tlh,* only a single case of *V. parahaemolyticus* was confirmed to have originated from a CT source.

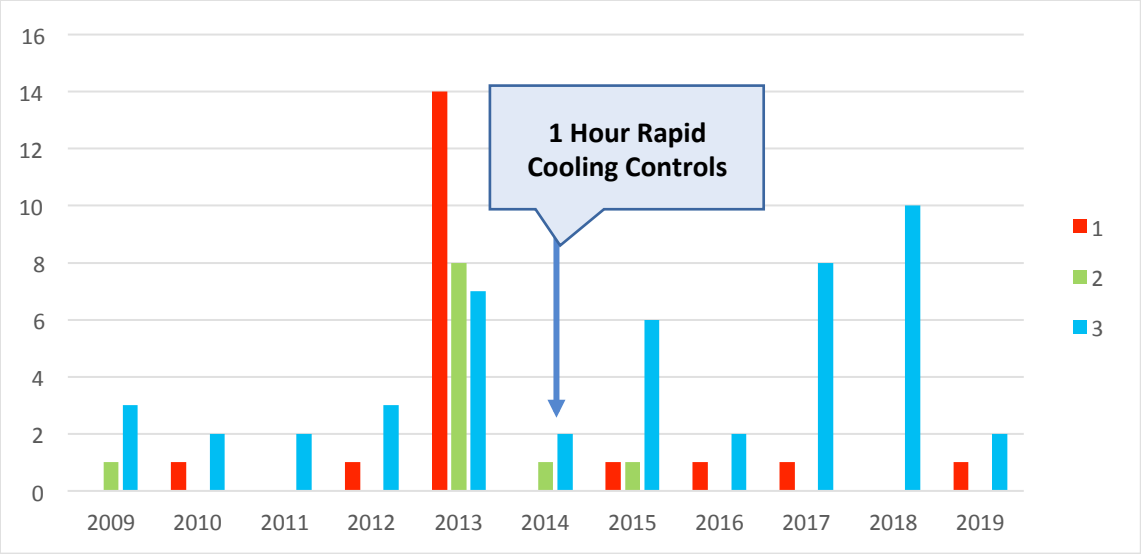


Figure 17. Connecticut *V. parahaemolyticus* illnesses 2009 through 2019. Traceback code 1 (red) are confirmed single source from CT, code 2 (green) are confirmed CT source, but more than one possible harvest location, and code 3 (blue) are cases with a multi-state traceback. Rapid cooling controls were instituted in 2014 in Connecticut.

This study did confirm that while *tlh* can be elevated even in background samples (Fig. 8), the standard pathogenic markers, *trh* and *tdh*, are consistently low (under 100 MPN/g) until exposed to ambient air for 5 to 12 hours. This is strong evidence that control plans allowing more than 5 hours for oysters to be cooled to an internal temperature of 50°F may significantly increase the risk of illness due to the increase in pathogenic strains of *V. parahaemolyticus.* A more extended cooling may help to decrease shellfish mortality due to rapid cooling. In the absence of mortality, rapid cooling controls of 1 hour are still recommended, however with additional local and regional pathogenic strain and illness data, managers may consider safely extending the cooling period to allow up to 5 hours to cool shellfish to an internal temperature of 50°F.

It is informative to consider states across a region to discern regional applications to harmonize management approaches. If NH only considered conditions within the state there would be no concerns given the rare incidence of illness, and MA would not realize that certain areas in the state appear to be regional and not only state hot spots. The approach used by Whistler to construct a regional framework for assessing risk from regionally significant pathogenic strains is a good example of how a regional approach can be useful. First, it required collaboration with both resource management and public health agencies in each state to gain access to clinical strains and the traceback information required to discern differences in strain prevalence and severity as pathogens. Pooling results from all states provides information on which strains are regionally significant. In the New England area, the results were striking in that 70-80% of illnesses were being caused by only two strains, making design of detection methods a doable task. This study was the first to apply these new detection methods to environmental samples in real time, revealing some challenges that will require further work this year as part of a related ongoing project.

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