Pacific Shellfish Institute Final Report to ISSC
"Techniques and Practices for Vibrio Reduction"

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This report covers the entire contracted period of October 1, 2014 through August 31, 2016. All studies funded by Interstate Shellfish Sanitation Conference (ISSC) in 2015 are reported here.

Figure 1. Portion of Hama Hama tidelands as seen from Highway 101 (top). Hama Hama tidelands (lower left) and an on bottom deep water cage (lower right).

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Background:

The Pacific Shellfish Institute (PSI) has been engaged in laboratory and field experiments focused on *Vibrio parahaemolyticus* (*Vp*) for over a decade, operating on the general hypothesis that elevated *Vp* levels in intertidal cultivated shellfish can be reduced by exposure to ambient water conditions. Submergence of oysters infected with *Vp* in cooler deep water theoretically suppresses *Vp* infection rate and development. This environment can drive purging of the bacteria from infected oysters. Although still preliminary, results detailed here validate this assumption. These results will assist in advancing farm based *Vp* reduction/avoidance techniques.

Based on the result of collaborative studies by Taylor Shellfish, we altered our proposed study design emphasizing using deep water intakes at oyster hatcheries for submergence and depuration. As Taylor Shellfish experimented with holding oysters in tanks filled with pumped cool deep water at their Quilcene hatchery in northern Hood Canal, the company realized the practice was not suitable at the farm scale. Water temperatures were not reliable and most oyster harvest occurred hours away from the deep water intake facility, therefore adding possible heat exposure time and increasing costs. Taylor Shellfish subsequently began pursuing deep water submergence, similar to PSI experiments with the Hama Hama Shellfish Company reported here (Figure 1). Concurrently with these studies we examined the ability of icing prior to shipment of oysters to suppress the development of *Vp*.

In addition to testing the potential of submergence to reduce *Vp* infection and development, we conducted preliminary tests of the technique to reduce levels of the saxitoxin in oysters that causes Paralytic Shellfish Poisoning (PSP).

Submergence Study:

This ISSC study was based at the Hama Hama Oyster Company, near the town of Lilliwaup, Washington. The study site is located at 47.535° N latitude and -123.039° W longitude in central Hood Canal (Figure 2).

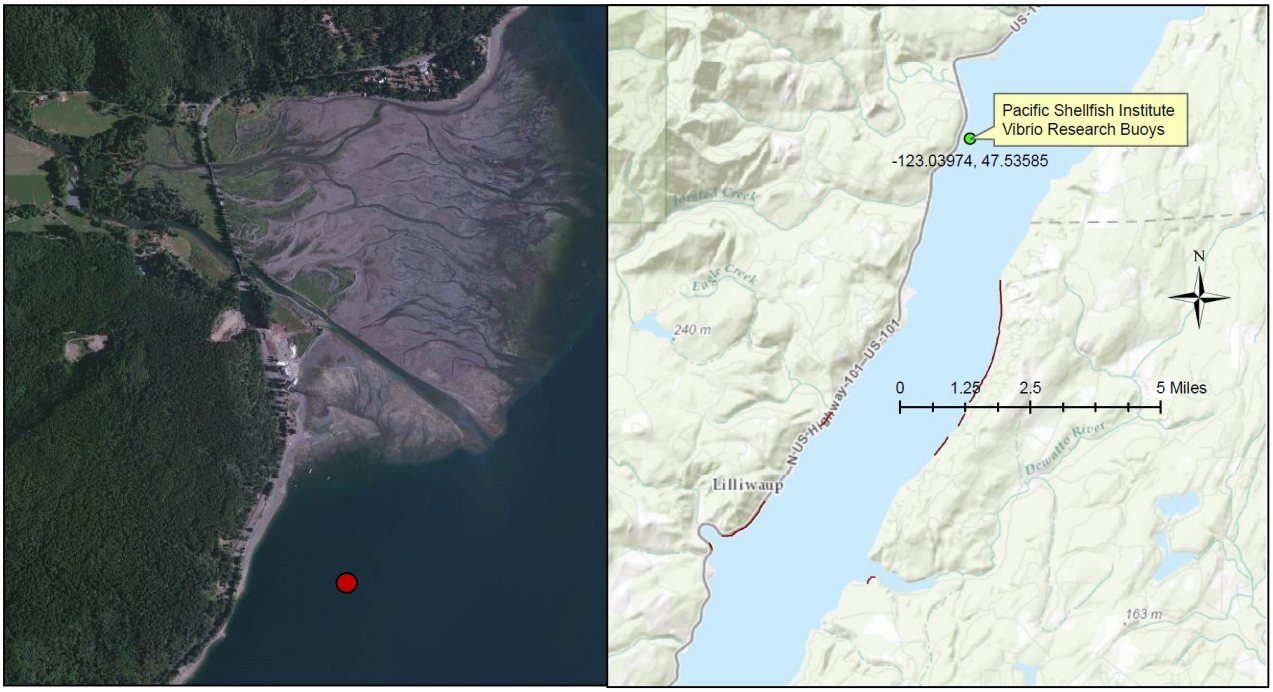

Figure 2. Hama Hama deep water holding area (red dot on aerial photograph) in relation to the farm/delta, and the broader region of central Hood Canal (green dot).

2015

During the summer of 2015, a total of 5 separate trials were conducted to evaluate the effectiveness of deep water holding. At the beginning of each trial, an average of 15 dozen market size oysters were transferred from intertidal growing areas to deep water depths of 35 and 55 feet. Water and air temperature data was recorded at submerged and intertidal sites using Onset HOBO® data loggers. For each sampling, a total of 3 replicates of 10-12 oysters each were collected, surrounded by gel ice inside styrofoam coolers and shipped to the lab for total *Vp* analysis. All oyster samples arrived at the laboratory in less than 24 hours. This shipping methodology is similar to the Washington Department of Health protocols on *Vp* sampling. Internal temperatures were measured from 4 sacrificed oysters during each collection prior cooling. *Vp* levels reported here are Most Probable Number (MPN) total *Vp* per gram, following BAM chapter 9 methodologies performed by AmTest Laboratories in Kirkland, Washington.[1] The commonly used BAM biochemical method was utilized to enrich, isolate, and enumerate the species.

[1] As previously planned, and reported to ISSC in June 2015, PSI utilized the services of AmTest Laboratories to be consistent with ancillary *Vp* studies being conducted in the region. During the summer of 2015 there

The purpose of Trial 1 was to evaluate whether oysters at these depths would accumulate *Vp* at a lower rate than those at the intertidal zone. Oysters were transferred on June 17, 2015 from Hama Hama Oyster Company intertidal oyster bed "S2" to 35' and 55' depths just south of the farm. Samples were collected at deployment and 15, 20 and 30 days after deployment, arriving at the lab within 24 hours. Results from this study are promising, showing on average that oysters at both depths had *Vp* levels 1.5 to 1.9 log lower than intertidal oysters (Figure 3).

Temperatures at deployment depths ranged from 51-52°F at 55' and 52-54°F at 35' (Figure 4). These temperatures are optimal for *Vp* purging according to lab-based studies conducted by Dr. Su at the Oregon State University Seafood Laboratory.[2] Specifically, Dr. Su's studies found that depurations worked best at 7°C /45°F to 15°C/59°F, while 22°C/68°F worked, but with less *Vp* reduction and 5°C was too cold for active feeding.

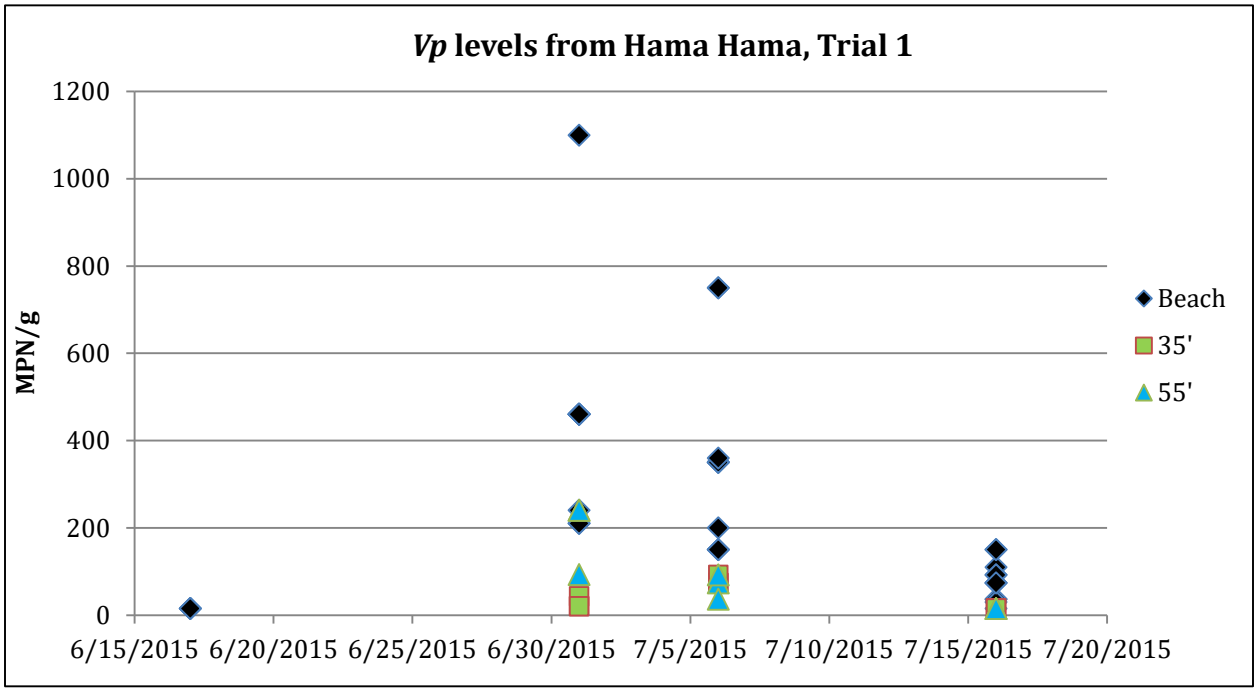

Figure 3. *Vibrio parahaemolyticus* levels in oysters harvested from suspended depths (35' and 55') and the intertidal zone (beach).

was consensus among PSI's collaborators, including the Washington Department of Health, that measure of total *Vp* was desirable, as opposed to TDH-related hemolysin hemolysin (trh+) and thermostable-direct hemolysin positive (tdh+) *Vp*. FDA staff were also consulted on the issue and agreed.

[2] Phuvasate, Sureerat; Chen, Ming-Hui and Yi-Cheng Su. 2012. Reductions of *Vibrio parahaemolyticus* in Pacific oysters (*Crassostrea gigas*) by depuration at various temperatures. Food Microbiology 31, pp 51-56.

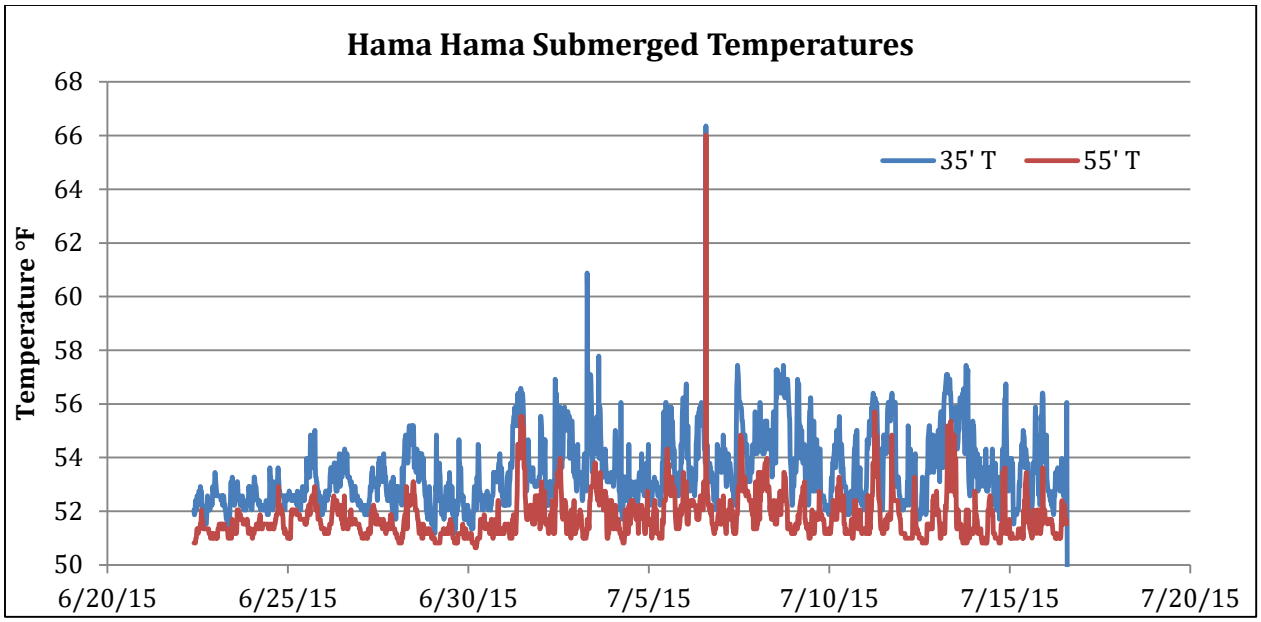

Figure 4. Temperatures at 35’ and 55’ depths during Trial 1.

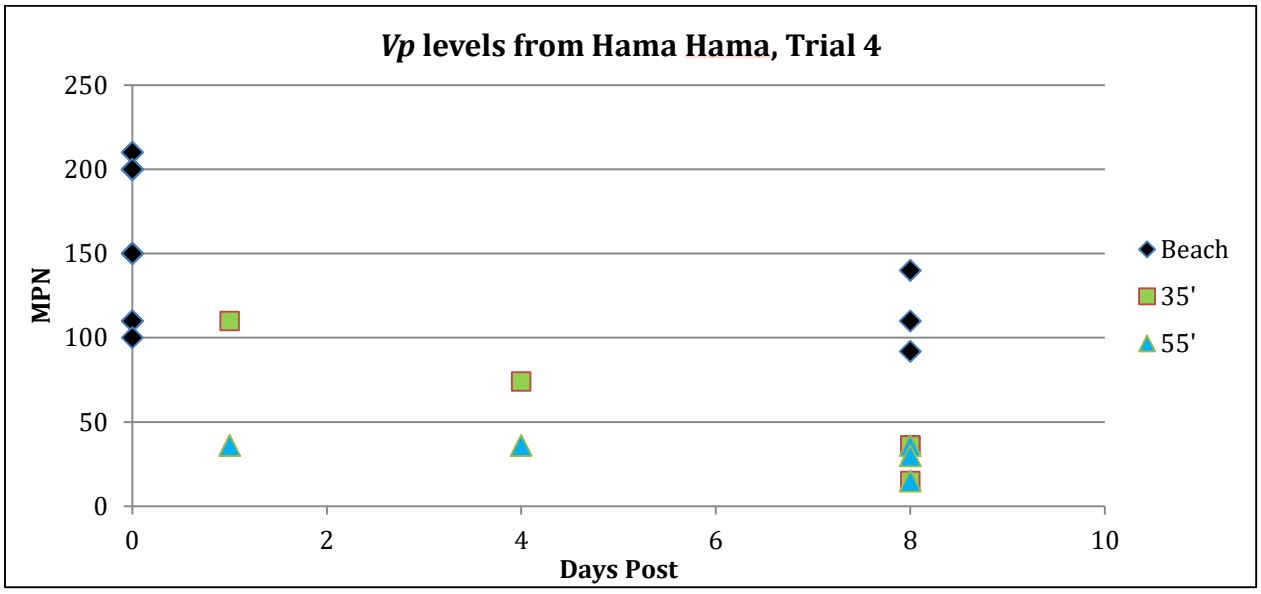

Figure 5. *Vp* levels of temperature abused oysters (Day 0) held at 35’ and 55’ compared with intertidal oysters.

While trial 1 examined potential increases in *Vp* in oysters deployed at depths of 35’ and 55’, trials 2 through 5 examined the effectiveness of reducing *Vp* in temperature abused oysters by deploying them at these depths. In an effort to find oysters with high background *Vp*, Washington Department of Health (WDOH) Shellfish Program data was utilized. Oysters were collected from three sites with WDOH reported high numbers of *Vp*: Jackson Cove in Dabob

Bay, northern Hood Canal (47.740° N -122.867° W), Pickering Passage in southern Puget Sound (47.264° N -122.923° W) and Hama Hama (47.535° N -123.039° W). After collecting 3 dozen for immediate lab analysis remaining oysters were deployed to depths of 35' and 55'. Oyster *Vp* levels were measured at 1, 4, and 8 days after deployment at both depths and when tides allowed at the collection beach. Background levels of *Vp* in temperature abused oysters did not achieve the desired level of >10,000 MPN/g, but a significant reduction of *Vp* was observed as early as 1 day post deployment in oysters held at 55' and at both depths by 3 days (Figure 5).

2016

Remaining funds from 2015 allowed for 2 additional trials to be conducted at Hama Hama. PSI staff closely monitored the seawater temperature data provided in near real time by the Washington State Department of Health (WDOH) and predicted weather in search of ideal *Vp* conditions. Staff also monitored WDOH reported *Vp* levels at other sites in Washington and determined that levels at Hama Hama should be ideal under the right conditions and temperature abuse. Samples collected by DOH on 7-28-16 were just below the action level of 10,000 MPN/g (Figure 6) without temperature abuse. Area 5 of Hood Canal was closed on 8-23-16 to 9-7-16 due to a sample collected on 8-21-16 at Hama Hama reading 24,000 MPN/g, 14,000 MPN/g above the action level.

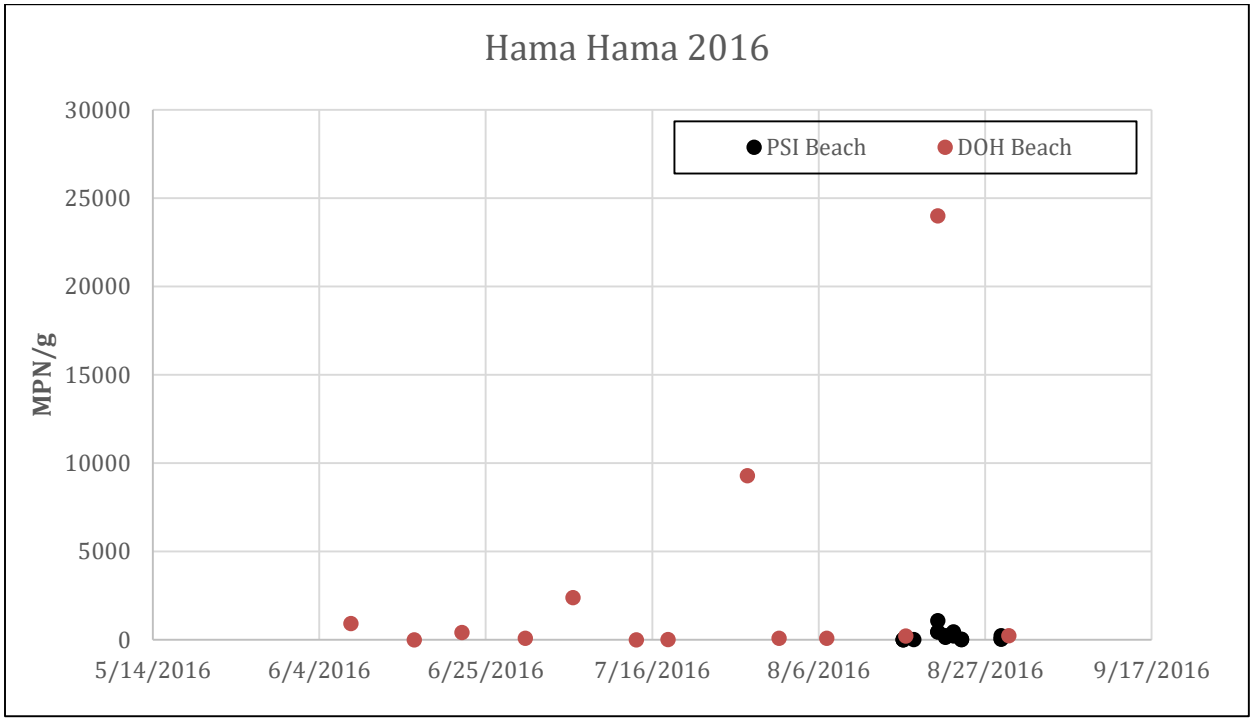

Figure 6. *Vp* levels of intertidal oysters collected at Hama Hama either by WDOH or PSI staff.

Trial 1 was initiated on 8-16-16 during a warm weather period in Hood Canal with increasing water temperatures measured at a 3' depth by WDOH. Oysters were collected intertidally and temperature abused by holding them on a concrete loading dock for 3 hours post harvest, so that oyster internal temperatures exceeded 34 °C / 93.2 °F. Oysters were sampled in replicates and shipped to Am Test Laboratories on gel ice as above. Remaining oysters were deployed at depths of 50' in either suspended cages or on bottom. Temperature data was collected at each deployment site and averaged 52.5°F on bottom and 52.9 °F suspended (Figure 7).

Despite the initial temperature abuse, targeted high levels of *Vp* were not achieved in these oysters, although levels were higher in subsequent intertidal samples taken on 8-21-16. While initial levels of *Vp* were not as high as anticipated, submerged oysters from subsequent sample dates had lower *Vp* post treatment levels than in oysters sampled intertidally the same day (Figure 8).

Trial 2 was started on 8-21-16 during a warm weather period; water temperatures exceeded 21 °C / 70°F. Oysters were temperature abused on the beach, harvested just prior to water coverage, and held upland for an additional 2 hours prior to deployment and sampling. Internal oyster temperatures recorded on the beach averaged 26.8 °C / 80.2 °F. Resulting *Vp* levels in the temperature abused oyster were slightly elevated, with a high of 1,100 MPN/g (Figure 10). As mentioned above, a WDOH collected sample during the same day reached 24,000 MPN/g, closing the area to oyster harvest until 9-7-16.

Temperature data was collected at each deployment site and averaged 53°F on bottom and 52.8 °F suspended (Figure 9). *Vp* levels were lower in oysters collected from submergence at 1, 2, and 3 days post-harvest compared to pre-submergence levels and in oysters collected on the beach the same day during low tide exposure. This was also true at 8 days post-harvest for oysters held on bottom at a depth of 50' but not for those held suspended at 50' which had *Vp* levels higher than those collected intertidally.

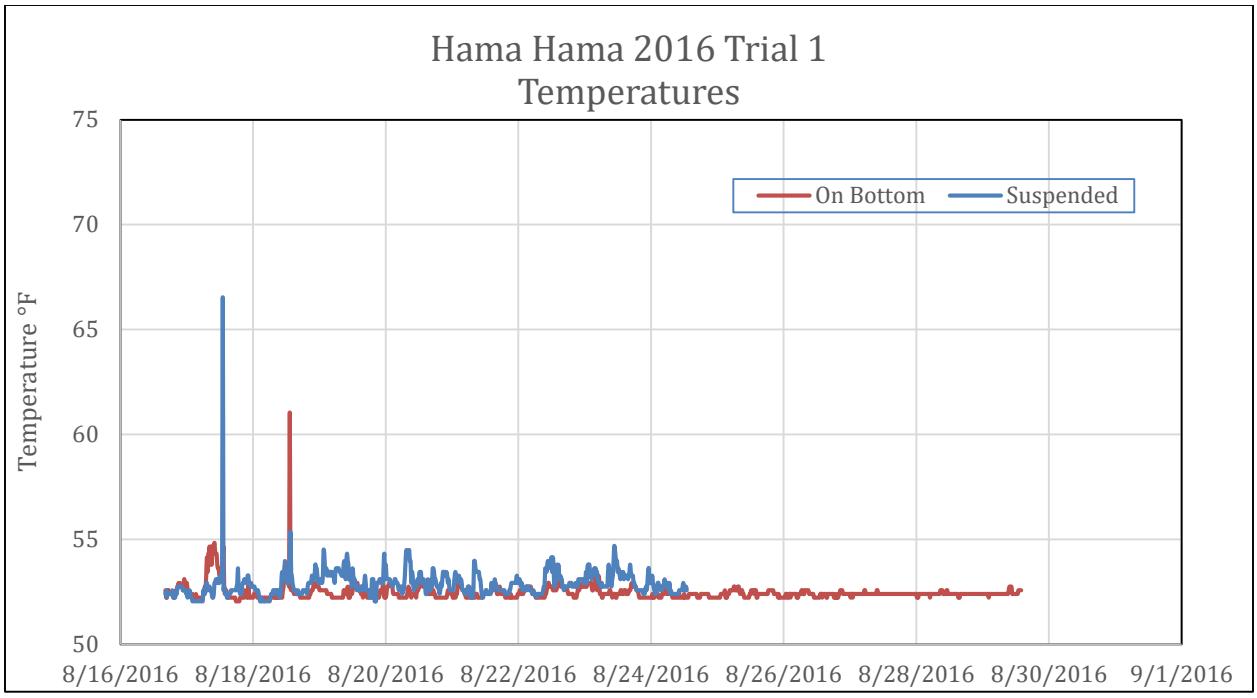

Figure 7. Temperatures in suspended and on bottom oyster cages during Trial 1.

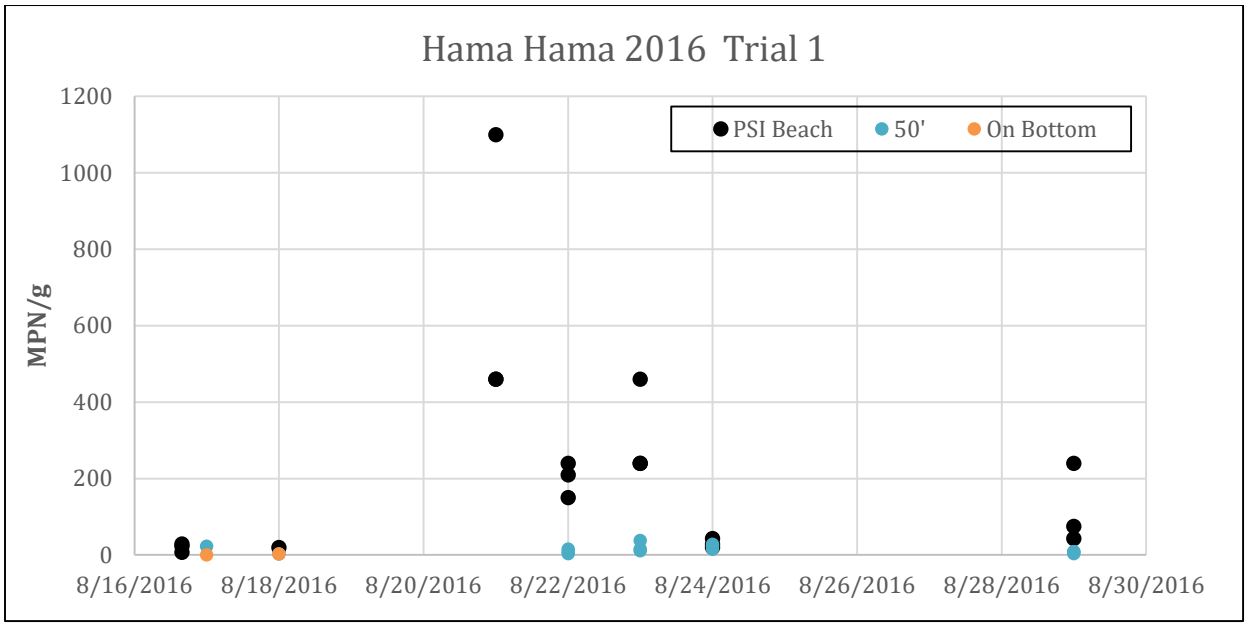

Figure 8. *Vp* levels of intertidal and subtidal oysters collected at Hama Hama by PSI staff during Trial 1.

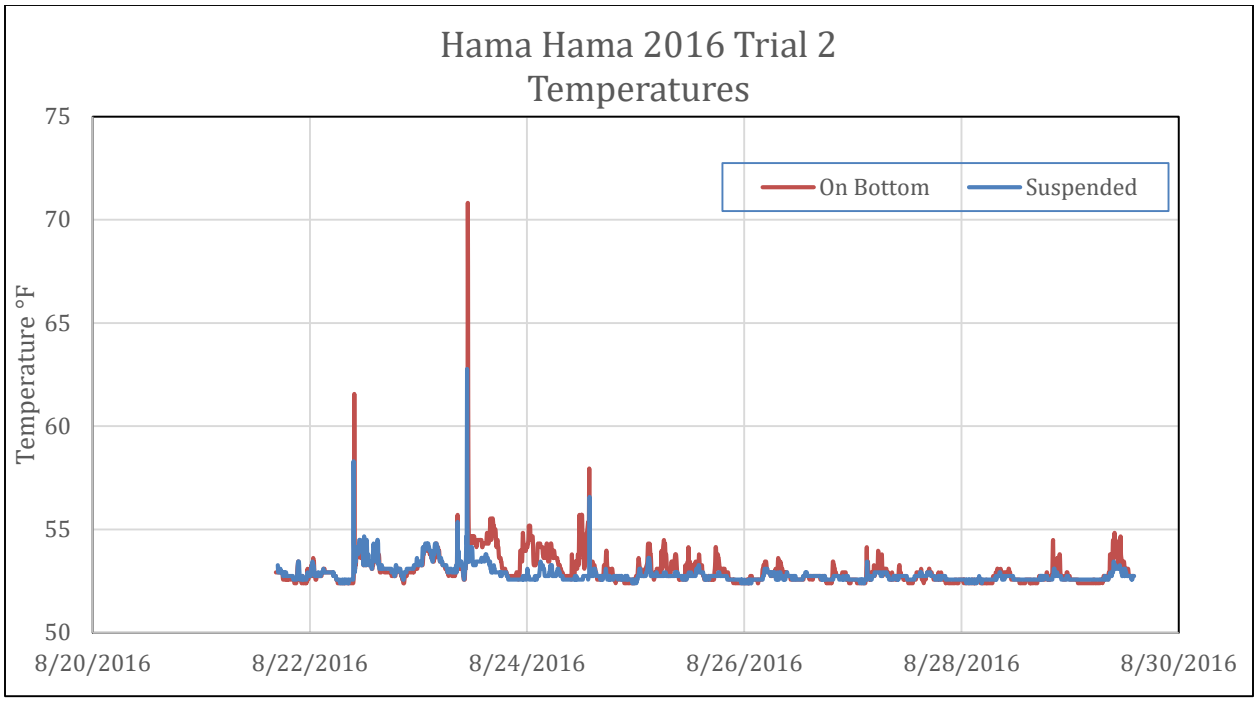

Figure 9. Temperatures in suspended and on bottom oyster cages during Trial 2.

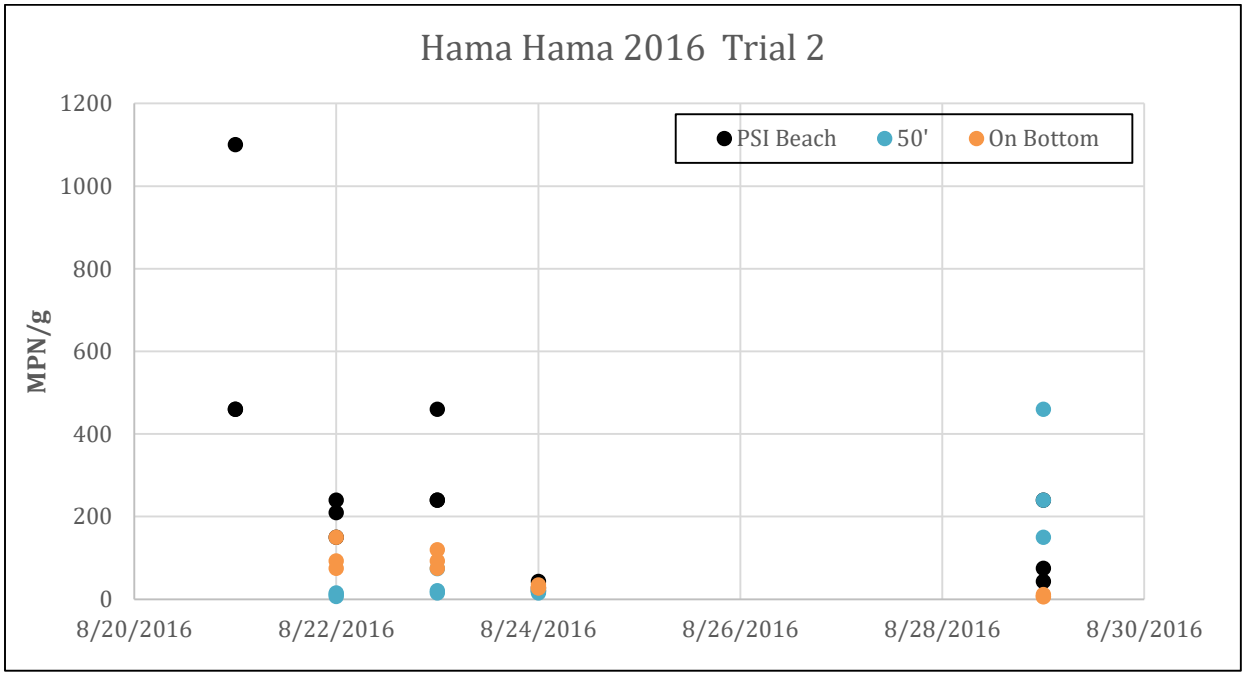

Figure 10. *Vp* levels of intertidal and subtidal oysters collected at Hama Hama by PSI staff during Trial 2.

Paralytic Shellfish Poisoning:

Paralytic Shellfish Poisoning (PSP), caused by the dinoflagellate *Alexandrium catenella* was prevalent in Hood Canal during 2015. Counts of *A. catenella* were extremely high and caused closures in growing areas Hood Canal 1, 2, 5 and 6, as well as Quilcene and Dabob (Figure 11). Partnering with the WDOH, the PSI and Hama Hama study team decided to see if PSP levels differed among the oysters held at depth. While sampling for *Vp*, samples were also collected and shipped to WDOH for saxitoxin (PSP toxin) analysis using existing lab capacity.

In contrast to the *Vp* studies, oysters held at 30' contained the most PSP toxin. Concentrations were an order of magnitude more than the intertidal oyster (Table 1). Oysters held at 55' did not contain a detectable amount of the toxin, which may serve as a side benefit of deep water holding. The results make sense, due to the increased access on phytoplankton at the 35' depth and decreased access at 55'. This small experiment should be replicated and validated before being used to avoid PSP.

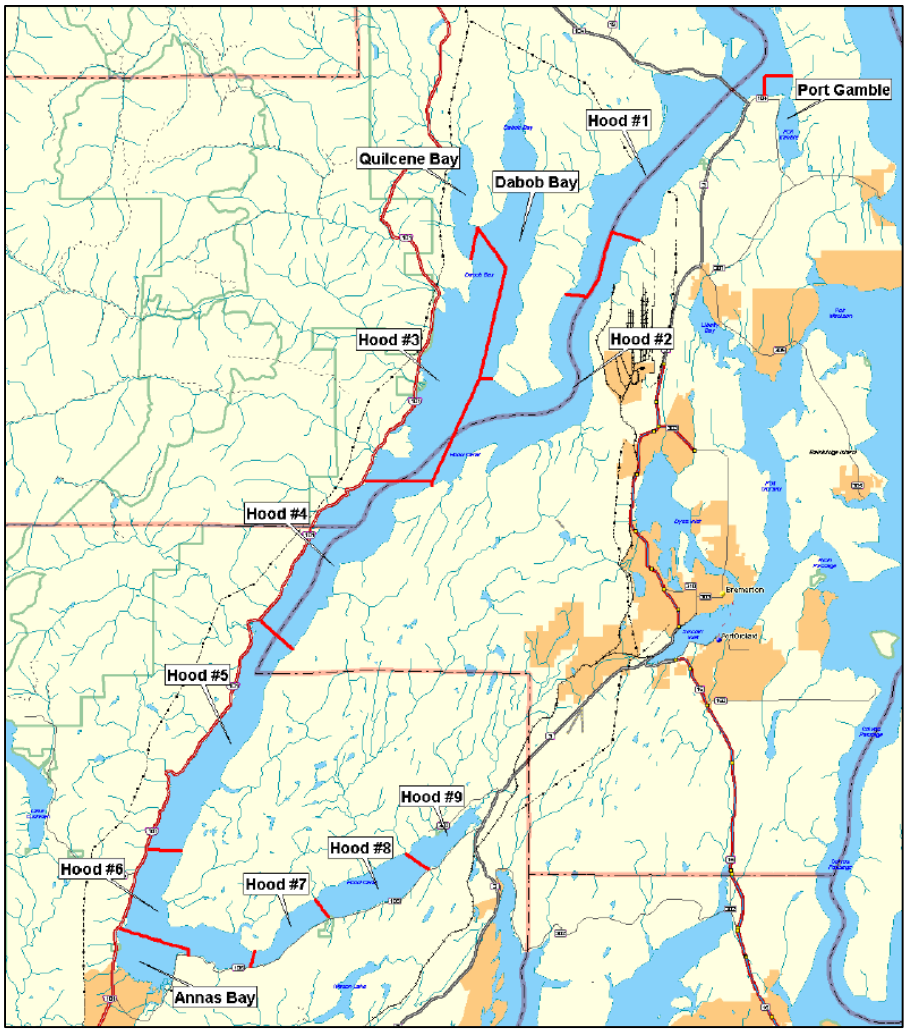

Figure 11. Hood Canal growing area map (modified WDOH map).

Table 1. Saxitoxin levels in oysters from the intertidal zone and at depth (35' and 55').

	Intertidal	35'	55'
6/25/2015	146		
7/6/2015	77	735	<38
7/16/2015	<38	130	<38

~ug/100g Saxitoxin

Shipment Method Trials:

In tandem with the 2015 trials, the effectiveness of icing prior to shipment to suppress *Vp* development was compared to conventional shipment in gel pack lined coolers. During collection of *Vp* infected oysters for relay to Hama Hama for the submergence trials, an additional 3 dozen were subjected to an ice slurry to reduce their internal temperatures below 4°C / 39.2 °F prior to gel pack shipment. *Vp* levels after shipment (Overnight FedEx or employee vehicle) to the laboratory were compared with levels in oysters handled in ambient temperatures prior to gel pack shipment.

When time permitted, additional oysters (3 dozen per treatment) were collected prior to low tide exposure and were either immediately iced or put in a cooler with gel ice. Oysters that were immediately iced were cooled to 4°C / 39.2 °F then put in containers with gel ice. All oysters were then shipped for analysis. A total of 4 trials were completed on separate days.

The *Vp* levels of the iced oysters were elevated compared to the direct shipped oysters in the first 2 samples by log 1.64 and 1.12, but levels were slightly reduced in the last 2 iced oyster samples by log 0.35 and 0.52. Analysis of variance (ANOVA) tests conducted on each trial concluded that *Vp* levels in iced vs direct shipped oysters were significantly different only in the first trial (Figure 12). The lack of significant difference is likely due to the low levels of *Vp* encountered as high temperatures and temperature abuse did not dramatically increase *Vp* levels (Figures 13 and 15).

As displayed in Figures 13, 14 and 15, no significant difference was found in oysters iced prior to shipment and those only placed in gel pack lined coolers. This is explicitly shown in Figure 13. There was a significant reduction in *Vp* levels in oysters collected prior to low tide exposure during the 7-6-15 trial (Figure 13). No significant reduction was observed in the 8-2-15 trial, when the average for all study groups was below 200 MPN total *Vp* per gram (Figure 15).

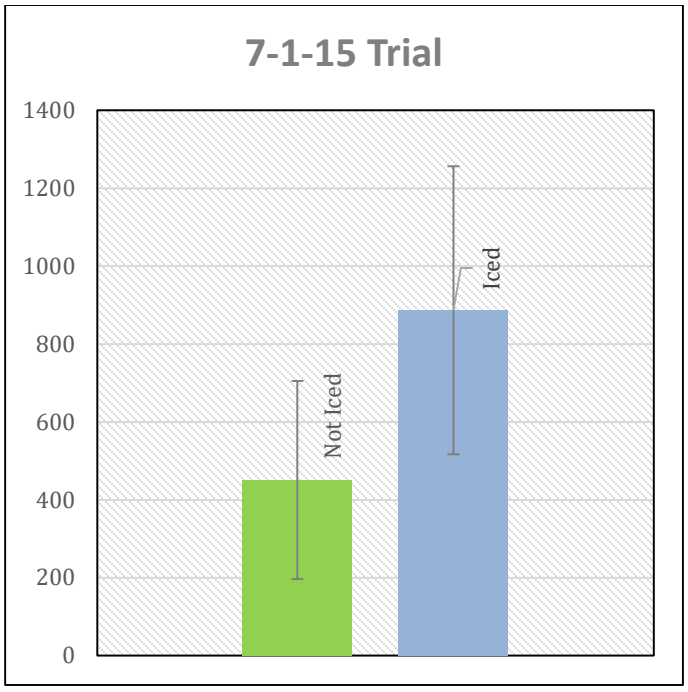

Figure 12. MPN/g total *Vp* in non-iced oysters and those subjected to ice slurry on 7-1-15.

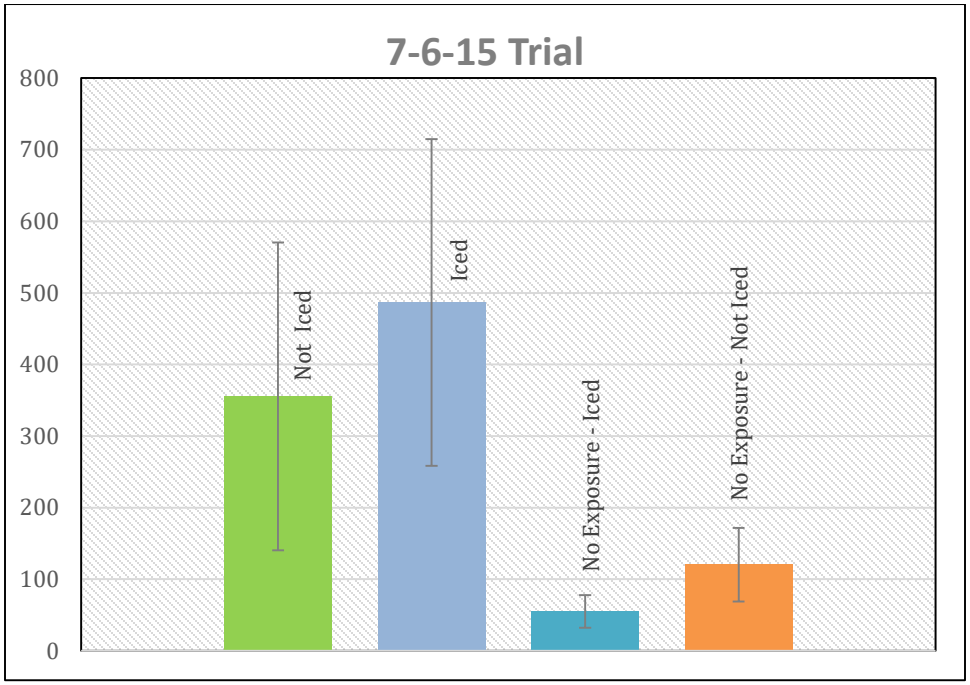

Figure 13. MPN/g total *Vp* in non-iced oysters, those subjected to ice slurry, those collected prior to exposure and iced and those collected prior to exposure and not-iced on 7-6-15.

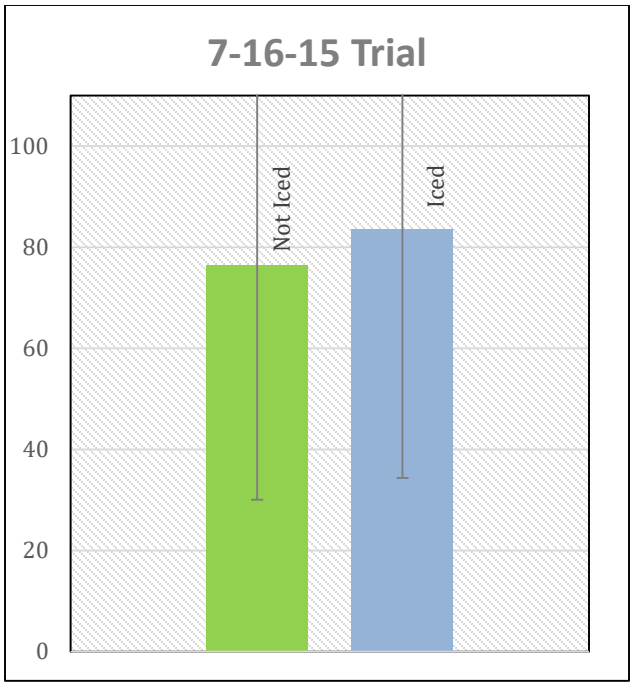

Figure 14. MPN/g total Vp in non-iced oysters and those subjected to ice slurry on 7-16-15.

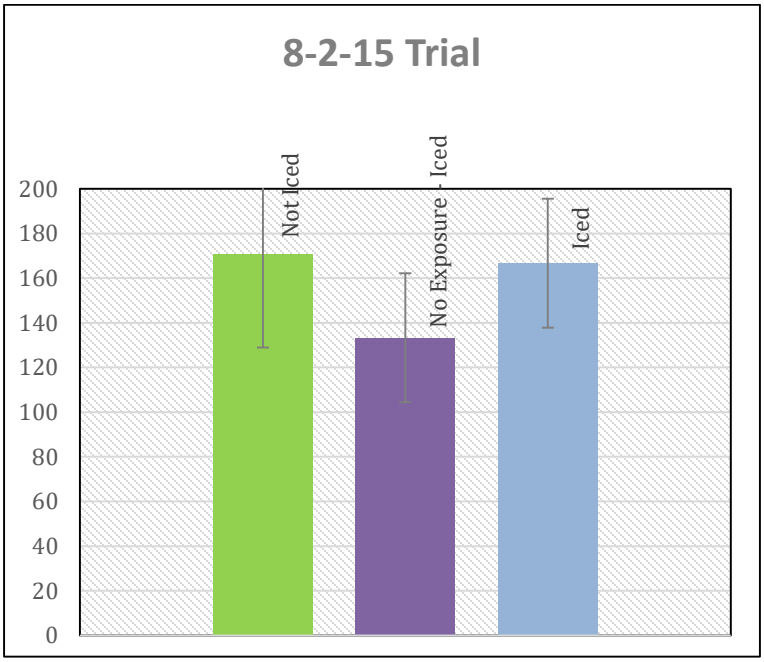

Figure 15. MPN/g total *Vp* in non-iced oysters, those collected prior to exposure and iced, and those subjected to ice slurry on 8-2-16.

Summary:

In summary, this research demonstrated that submergence of *Vp* infected oysters in cooler deeper water can suppress and potentially reduce levels of *Vp*. Additionally, hot weather and temperature abuse did not dramatically elevate *Vp* levels in these experiments, an issue other researchers in the area have dealt with. During summer of 2015 the background levels of *Vp* were low in Washington Department of Health samples, Taylor Shellfish Farms studies and the PSI studies detailed here. Rainfall was exceptionally low during the summer of 2015, which may account for the relatively low levels of *Vp* throughout South Puget Sound in that year, but background levels of *Vp* were also low in 2016 at PSI and Taylor Shellfish study sites in Hood Canal and Totten Inlet. The trials conducted in 2016 again reduced or avoided increases in *Vp* levels in oysters suspended at depths of 50' and held on bottom at 50'. There was no significant difference between oysters held on bottom or suspended.

The companion study analyzing levels PSP-causing saxitoxin demonstrated that the toxin accumulated at higher levels in the submerged oysters if suspended within the phytoplankton zone. Concentrations were lower in oysters held below the bloom. This finding may warrant consideration when planning sites for new shellfish farms.

Next steps:

The Hama Hama Oyster Company is located in the Hood Canal #5 WDOH shellfish area, which is currently in risk category 2. No single source *Vp* illnesses were attributed to Hood Canal #5 in 2016 so it will likely remain in this category. Category 2 requirements are detailed in Table 2. Study site temperatures recorded at depths of 35 to 55 ft during this 2-year study averaged 11.7°C / 53 F°, much lower than the action temperatures of 66°F and 68°F specified in the last two Category 2 requirements (Table 2). Oysters are allowed to be commercially contained and harvested at these depths if the oysters were initially collected from open areas. So, if oysters are collected right before a closure conditions at a particular beach they can be held and harvested at these depths. In other words, oysters held at these depths during the high air and water temperature summer-fall period, when beds are typically closed to harvest, could be harvest if temperatures are consistent with those found during this study.

Site permitting requirements by the United States Army Corps of Engineers, Washington Departments of Natural Resources and Fish and Wildlife, and Mason County will need to be met prior to commercial implementation. The current farm strategy will deploy oysters when water and air temperatures are below 66° F at the site of collection, avoiding oysters with high levels of *Vp*. A WDOH control plan waiver is not needed in this scenario. A control plan waiver would need to be obtained from WDOH to harvest oysters from a closed area to this deepwater site. If this option is pursued, more sampling may be required to test *Vp* levels of oysters held at these depths since the worst case scenario, high levels of *Vp* at deployment, was not seen during this study.

Table 2. Washington Department of Health Risk Category 2 Requirements (modified from www.doh.wa.gov/CommunityandEnvironment/Shellfish/CommercialShellfish/VibrioControlPlan on 12-20-16).

Risk Category 2 Requirements	Time to Cooling
Except as noted below, the time of harvest to cooling requirement from May 1 through September 30 is:	7 hours
When ambient air temperature at harvest is greater than 85 degrees F, the time of harvest to cooling is:	5 hours
When harvest temperature is between 66 degrees F and 68 degree F from July 1 through August 31, the time of harvest to cooling requirement is:	3 hours
Harvest Control: From July 1 through August 31, harvest is not allowed for 24 hours when harvest temperature is above 68 degrees F.	

Acknowledgements:

PSI thanks the staff at Hama Hama Oyster company for their assistance in study design, site logistics, oyster sampling and shipping. Staff include Adam James, Justin Stang and Louie Lakenes.

WDOH staff were invaluable in their assistance in providing study feedback, regional *Vp* sample numbers as part of their regular monitoring effort and in running additional samples for saxitoxin (PSP toxin) analysis. Specifically, staff members Clara Hard, Adi Hanein and Laura Wigand Johnson.

Finally, Kurt Johnson of Taylor Shellfish continued to be an important collaborator on study design, interpretation and occasional commiseration.