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Seed Health Research & Technical Training in New Oyster Seed Rearing Practices

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Photo: Seed Pacific Oysters reared in Legoe Bay Shellfish Hatchery

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Executive Summary

This project addressed the challenges shellfish farmers face rearing oyster seed. The limitation of oyster seed was cited by Pacific coast shellfish farmers to be one of the most serious constraints to regional industry growth. We examined the environmental and logistical factors that impact seed production in hatcheries, remote set facilities, and nurseries across the west coast. Additionally, we assisted growers with analytical and diagnostic support in seed-rearing facilities and participated in an educational information-sharing workshop for growers and nursery managers.

The Pacific Shellfish Institute (PSI) implemented a survey in Washington, Oregon, California, Alaska and Hawaii to characterize the techniques and challenges faced by oyster growers, and to identify the frequency, severity and source of hatchery-related mortality events. Results from the survey indicate strong commonalities amongst west coast nurseries in regards to seed rearing and setting practices. Nurseries additionally identified environmental factors (e.g. water quality and marine pathogens) as the primary inhibitor to production. Mortalities were ubiquitous amongst nursery operations, and were most strongly associated with cold temperatures, smaller seed sizes, poor water quality, and the presence of pathogens.

To better understand the environmental factors at various operations, PSI deployed two multimeter probes in North Puget Sound & Hood Canal to continuously monitor water quality conditions. Multimeters monitoring pH enabled hatchery staff to buffer and enhance sea water accordingly, and to time the drawing of water into their facilities. PSI rotated the instruments between facilities on a monthly basis to measure water quality parameters and carbon chemistry from water samples. Six companies utilized this service during the project period. The continuous, real-time data proved valuable to each producer by enhancing their understanding of water quality dynamics in association with diel and tidal cycles, weather patterns and seasons. Overall, the project increased the number of monitoring devices and access to site-specific data.

Analytics at nursery operations identified *Vorticella spp.* ciliates and *Vibrio spp*. proliferation as a source of seed mortality, as well as bacterial contamination of algae mothers. Diagnostic exams performed by AquaTechnics indicated overwhelmingly that seed oysters were underfed, leading to death in nurseries and/or shortly after out planting to the farm. Due to the high occurrence of mortality in adult Pacific oysters in Washington over the 2018-2019 summers, project funds were adapted to assist growers in diagnostics testing of adults, in addition to larvae and seed oysters. Causes for adult mortalities were not frequently linked to a single source, but were assumed to be a combination of stressors including temperature and pathogen effects.

Pacific Hybreed initiated research on the design, construction and resting of a novel combined, setting and primary nursery system for potential use by the Pacific coast shellfish industry. The early post settlement phase of production of Pacific oysters is a common point of losses, contributing to declines in oyster seed availability. Success or failure with remote setting larvae for Pacific oysters essentially depends on the health and condition of larvae being reared for setting, thus increased scrutiny and a better understanding of the quality and quantity of live

microalgae was critically needed. As such, Dr. Joth Davis with Pacific Hybreed compiled a review of pertinent literature to consider industry oysters rearing practices, in order to optimize post set survivorship. The review focuses on published and unpublished firsthand findings that have been largely ignored or forgotten by hatchery operators on the Pacific coast, with specific reference to existing approaches for setting and rearing small seed for Pacific oysters.

Goals and Objectives

The overall goal of this project was to address the immediate needs of the shellfish industry for: 1) enhanced understanding of seed survivability in small- to mid-scale nursery and remote setting systems, and 2) training for small and medium shellfish farms regarding best practices for remote larvae setting and early post-set seed rearing. The following objectives were identified:

1) To conduct a cooperative industry oyster seed survival and performance assessment, with environmental analysis.
2) Survey post-set oyster seed health and disease at nursery and remote setting facilities in Washington, Oregon, California, and Alaska.
3) Evaluate short-term effects of food limitations/stress on seed oysters, and establish initial criteria for feeding and density production standards.
4) Inventory and evaluate new and existing systems for remote setting and early post-set seed rearing.
5) Conduct research on remote setting and post-larval seed culture methods for 2-3 new and/or existing systems to:
    a. refine methods for rapid on-site diagnosis,
    b. establish environmental parameters, feeding tables for species and size progressions, and
    c. establish on-site criteria for seeking laboratory assistance.
6) Host a "Remote Setting and Nursery Culture for Shellfish Growers" workshop to provide findings from Objectives 1-5 and technical training on seed rearing methods, micro-algae culturing, and water quality monitoring.

Industry Engagement: identifying common practices and needs

Outreach and Industry Participation

Negative effects on seed are not always explained, but are recognized to have significant impacts on the oyster industry. In 2013, Pacific Coast Shellfish Growers Association (PCSGA) convened an emergency meeting to address the threats to seed health and viability on the west coast. All sizes of shellfish growing operations expressed the need to understand the factors critical to the survival of oyster seed through research and training.

Project investigators engaged the industry in an effort to assess the status of seed rearing and current needs on the west coast. To reach seed producers, Pacific Shellfish Institute (PSI) and Washington Sea Grant (WSG) held a joint workshop titled "Remote Setting: lessons learned and future needs" at WSG's Conference for Shellfish Growers in 2017 in Union, WA. This workshop helped identify industry priority needs, provided a forum for inner-industry discussion, and allowed researchers to communicate their services for producers facing challenges in their facilities. PSI distributed a survey to workshop participants that served as a means to categorize common experiences and practices on the west coast to better understand the challenges and successes in regional seed rearing.

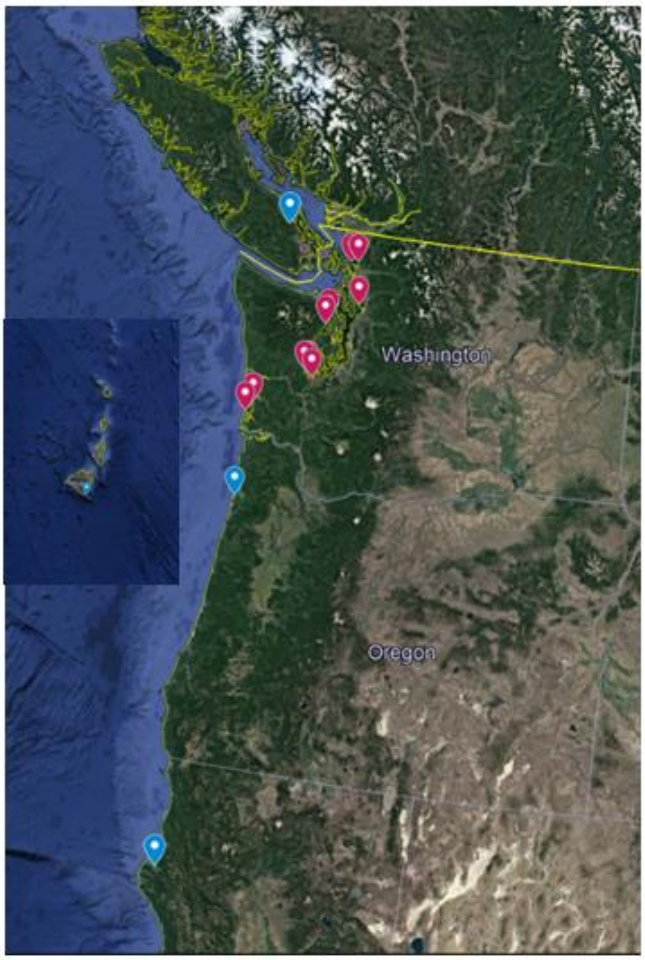


Fig 1. Survey participant locations spanning California, Oregon, Washington, British Columbia, Hawaii.

At the setting workshop producers learned from Dr. Ralph Elston's observations and suggestions from his career as a shellfish pathologist. With Dr. Elston, producers discussed methods of sanitation, because materials or pathogens can interfere with the setting process (most noticeably by preventing setting and/or causing mortality in the early life stage [<1mm; 8-10 days]). Baselines of feed, temperature and salinity were also addressed, with an emphasis on the common reality of underfeeding (which can often lead to disease-prone seed). Shellfish growers were able to openly share techniques that improve their success, as well as challenges they have faced. Overall, growers were interested in having additional workshops, noting that it would be especially valuable to have hands on training on-site. Dr. Elston and PSI advertised their availability to assist facilities, including monitoring and diagnostics relating to water quality, phytoplankton, and pathology for interested parties.

To more fully engage industry outside of Washington, surveys were provided to hatchery, nursery, and remote setting facilities in California, Oregon, British Columbia, Hawaii and Alaska. A total of 18 facilities provided information across the region, with the exception of Alaska. The facilities targeted by the survey ranged from small scale (two employees) to large scale (up to 30 employees). Both seasonal production, such as remote set facilities, and year round production (e.g. hatcheries) participated in the survey. Facilities reported a wide range of

production of single oysters: from half a million to 40 million per year. Cultch bag production from remote set facilities ranged from 40 to 80,000 units of cultch. Six of the survey participants sold larvae, seed and cultch. The majority of suppliers of larvae and seed to west coast hatcheries were located in Hawaii, followed by Oregon, which is home to an important Pacific oyster production facility.

a) b) c)

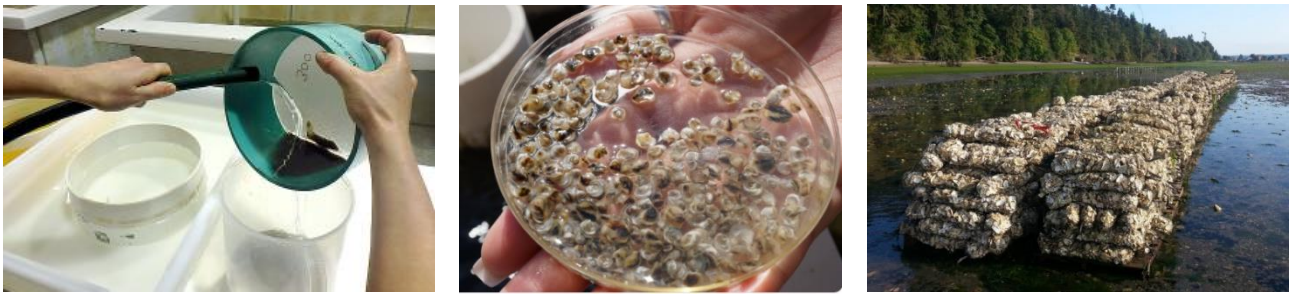


Fig 2. a) Larval Pacific oysters from Nova Harvest Ltd in Bamfield, B.C., Canada (www.novaharvest.com). b) Pacific oyster seed from Taylor Shellfish Hatchery, Quilcene, WA (www.taylorshellfishfarms.com/seed-sales) c) Post-set Pacific oyster standard size cultchbags from Rock Point Oyster Co., Quilcene, WA (www.rockpointoyster.com).

Setting/Rearing Techniques

West coast facilities were surveyed for a variety of conditions and techniques common to production in order to gauge a baseline for practices amongst the region's producers, identify best practices, and identify gaps in technology and training. Workshop participants reported improved larvae set at higher temperatures. However, sophisticated energy input into a remote nursery system is required to raise water temperatures when setting in cooler months. Remote setting was therefore found to take place most commonly between the months of April and October, with the most common remote-set months being between June and August, corresponding with warmer water temperatures. Nursery facilities of all types rely on sourcing outside seawater. The range of temperatures at which facilities set larvae was from 20° C to 28° C. The preferred setting temperature, or mode, for those surveyed was 27° C, with an average setting temperature of 24° C. The survey additionally found that seawater temperature was maintained through larval settlement and metamorphosis in 94% of survey participants. No participants reported using any chemicals to enhance settlement.

There are a variety of Pacific oyster setting techniques in hatcheries (Fig. 3a). Among all techniques reported during this research, >75% of seed producers relied on microcultch downwellers (Fig 4a.), both with and without recirculating water. Aerated setting trays and flow through setting trays with no recirculation comprised the remainder of setting techniques in the hatchery. Seed rearing techniques were variable amongst producers (Fig. 3b), with the most commonly reported method of rearing settled oysters from spat to seed in the upwell/downwell system utilizing 20" diameter bins in a three silo upwell box (Fig 4b.). The next most common system was a Floating Upwell System (FLUPSY). Fluidize bottle system was the least popular seed rearing method.

a) b)

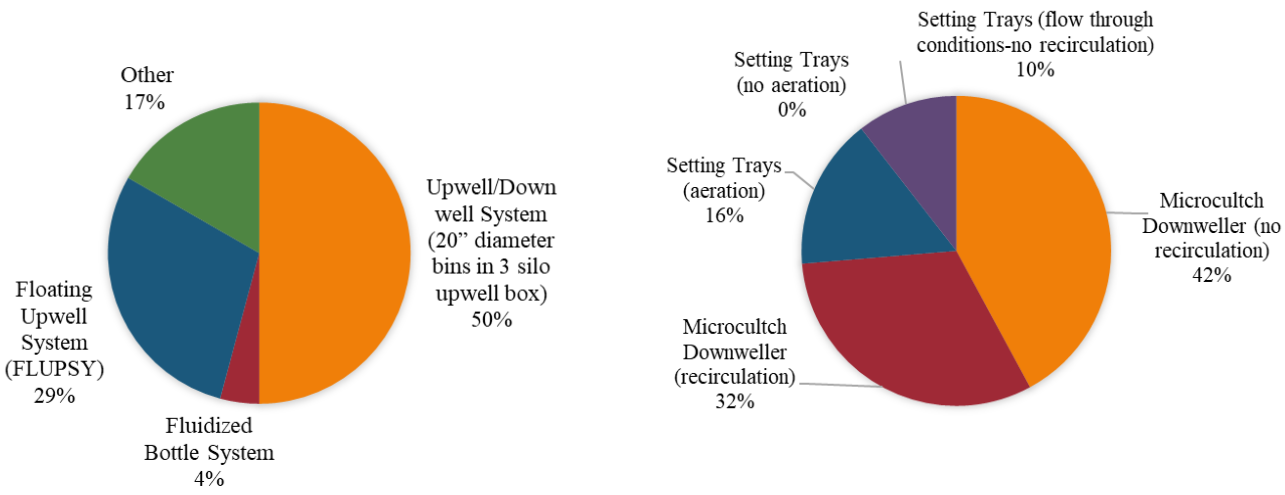


Fig 3. a) Percentage of survey participants that reported on oyster rearing techniques. The upwell/downwell system was the most commonly used by west coast producers. b) Percentage of survey participants that reported using the five pacific oyster setting techniques in the hatchery.

a) b)

Fig 4. a) Microcultch Downweller system from Nova Harvest in Bamfield, B.C., Canada (www.novaharvest.com). Microcultch downwellers are the most commonly preferred system by west coast hatcheries. b) Upwell/downwell system as pictured by Pangea Shellfish Company (Boston, MA) (www.pangeashellfish.com). This three-silo system is the preferred rearing method of west coast seed producers.

Challenges to Survival and Growth

Producers face a variety of environmental, technical, and labor-associated challenges when rearing seed. One of the largest challenges cited with seed and larvae production was environmental. Table 1 ranks the issues facing growers by most to least common. Water quality was the primary issue implicated in mortalities. Lack of equipment and lack of training also ranked high on the challenges list, along with permitting and logistics. Slow growth was the lowest ranked factor posing challenges to seed facilities. Shellfish producing companies that completed the survey also cited microbial contamination, lack of larvae supply, system crowding and bottle-necks relating to customer demand. Two producers stated that they were unable to determine the causative agent of their Pacific oyster mortality event.

Table 1. Challenges to oyster seed success and survival, faced by west coast seed producers.

Ranking	Challenge	# of Producers Citing Challenge in Survey (n=19)	% of Producers Citing Challenge in Survey
1	Environmental (water quality)	10	25%
2	Lack of Equipment/Gear	9	22%
3	Mortalities	6	15%
4	Lack of Training	5	13%
5	Permitting/Logistics	4	10%
5	Site/Location-Specific	4	10%
6	Slow Growth	2	5%

Mortalities in Pacific oyster seed facilities were reported by 14 (78%) of survey participants. Among participants that reported a season, 50% experienced mortalities in colder weather (April and earlier). Furthermore, 78% observed mortalities in smaller seed classes <1000 um. Producers offered up several explanations as to why they were seeing mortalities in their facilities. The most common issue was in-line with the most common reported challenge (Table 1): environmental parameters, including low salinity, low temperatures, low pH and high turbidity. The second most commonly cited reason for mortality was biological pathogens specifically viruses, bacteria, and amoeba. The quality and quantity of food was the third most observed by facilities to cause failures, followed by ciliate contamination. Some producers attributed "other" factors as significant to their oyster mortality events, including employee error and lack of training. Operators also referenced algal species, such as *Akashiwo sanguinea* and a flatworm as contributing to juvenile die-offs.

Some survey respondents also made reference to animal development and sensitivity observed during metamorphosis. These observations were similar to diagnostics by Dr. Ralph Elston, of Aquatechnics, during follow-up testing of mortalities in seed facilities during the project period. Dr. Elston found the following factors to be the major causes of mortalities (ranked in order of impact): 1) inadequate feeding, 2) contamination of feed, 3) invasive ciliates, and 4) Vibriosis. However, Dr. Elston notes that, recently, the frequency of Vibriosis detected in diagnostic examinations has been on the decline.

Successes and Future Planning

Technologies and practices have played an essential role in increasing growth and survival in juvenile Pacific oysters. Utilizing hatchery probiotics developed by Dr. Ralph Elston, has led to improved growth and survival for some seed producers. Seed producers reported a lower mortality risk with the use of probiotics during the 2017 "Remote Setting: lessons learned and future needs" workshop, and among survey responses. In general, seed facilities that invest in technology and staff dedicated to the observation, identification, and elimination of microbial contaminants have reported heightened success in survival at their hatcheries. There is a similar trend in demonstrated success for facilities that have invested in the infrastructure to produce algae on site. Producing algae at a hatchery facility improves the quality of food going to the larvae, and decreases costs and uncertainty associated with externally purchasing algae.

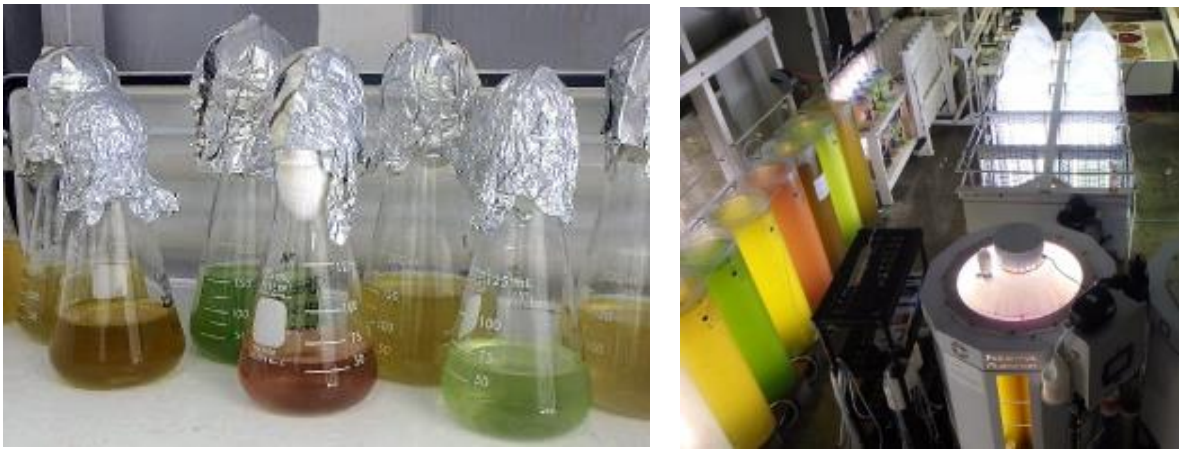


Fig 6. Algae production in the Nova Harvest Hatchery facility in Bamfield, B.C., from small-scale (left) to large-scale (right). On-site algae production has increased the quality of feed to juvenile Pacific oysters and reduced costs associated with purchasing algae from outside producers.

There was an overall desire from surveyed facilities (>60%) to increase their capacity for seed and/or larvae production. Adopting useful technologies such as FLUPSYs and UV sterilizers, and providing technical training to staff will aid west coast growers in moving forward with their production goals. The majority of producers plan to extend seed production well into the future, with a significant portion of these producers expecting to expand production. For many of the producers looking to expand, current facilities are at capacity, and expansion will depend on new or modified facilities. For some producers, future development and production hinges on addressing the biological and physical issues that face their water source. For smaller companies, this may mean seeking outside assistance in training or testing.

Regardless of the challenges that face producers at this time, or their future production goals, it was a universal sentiment from those surveyed that information sharing and discussions, such as the remote setting workshop held at the Washington Shellfish Grower's Conference, are essential for a better understanding of how the industry can continue to adapt to changing environmental conditions, technical challenges, market demands, and accessibility to dependable labor.

Theory to Practice: providing technical assistance to producers

PSI offered assistance to farms to respond to requests from west coast seed producers for on-site assistance and training. The majority of assistance provided included water quality monitoring, and the training to apply the water quality data to facility management. In partnership with Aquatechnics, PSI also assisted two small and mid-sized facilities in identifying biological contaminants, including invasive ciliates and bacteria in seed setting facilities. Assistance also resolved identified issues relating to quality and quantity of food provided to Pacific oyster seed. As facilities range in size of existing infrastructure and staffing, PSI's assistance scaled to the producer's needs, including scientific oversight to allow managers to facilitate their own investigations. The following case study provides a detailed example of technical assistance over the span of the project period for one Washington-based hatchery.

Small-Scale Farm Assistance: A Case study from Washington

Case Study: Legoe Bay Shellfish Hatchery

Facility Scale:	Small
Personnel:	3
Products:	Geoduck/Pacific Oyster Seed
Location:	Legoe Bay, Lummi Island, WA
Years in Operation:	6

The challenges seed producers face against environmental factors are ubiquitous. However, for small, relatively isolated hatcheries, such as Legoe Bay Shellfish Hatchery in the far Northwest of Washington State, it can be challenging to gain access to established monitoring networks and technical assistance that is more readily available for larger, more centralized growers. The small hatchery is owned and operated by Leah Paisano, who faces the challenge of maintaining production at her remote facility in the dynamic Rosario Strait, while facing the all too common issues of pathogen contamination and seed mortality. It is independent producers such as Leah that this project aimed to assist.

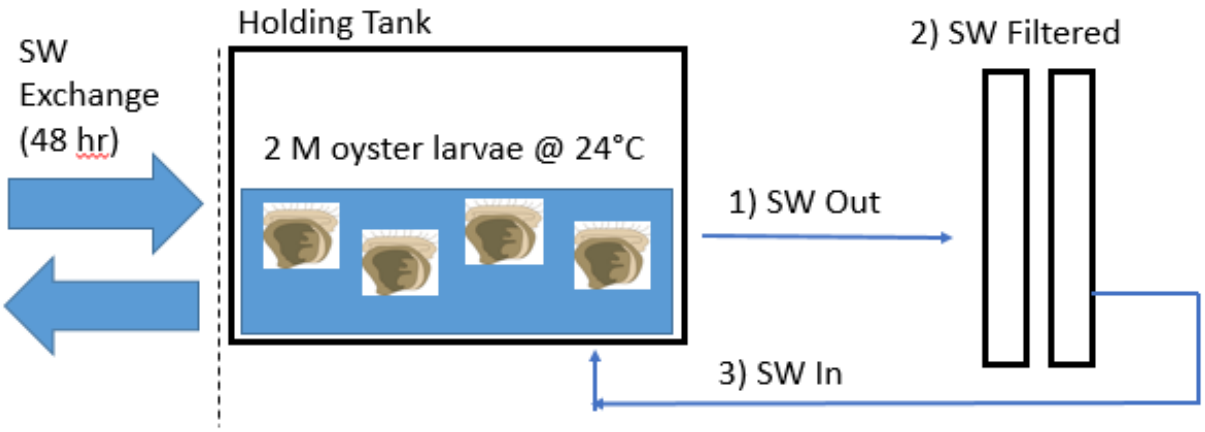
When Leah came to Pacific Shellfish Institute Sr. biologist Andy Suhrbier for assistance in 2014, she was having significant issues with pathogen contamination, specifically *Vibrio* bacteria and invasive ciliates. Partnering with Leah and Aquatchnics, Pacific Shellfish Institute designed tests that would determine the extent of the problem, and address the effectiveness of certain seed rearing and sanitation methods in maintaining healthy populations at her hatchery. The following summarizes the experimental procedures and results from the investigation.

Leah Paisano

Experiment 1: The Effect of Recirculated Water on Vibrio Bacterial Contamination

Objective: Vibrio pathogens can cause significant disturbances in hatchery systems, most commonly during summer months. This experiment addresses the ability of recirculation to control the levels of Vibrio in the system.

Design: Two million eyed larvae were put into a recirculating seawater (SW) system at 24°C for 5 days starting June 15, 2017. Larvae were fed daily and seawater was exchanged in the system every 48 hrs. Larvae condition and pathogen levels were assessed at the end of the 5 days.



Observations:
Day 1: 2 M eyed larvae introduced to recirculating seawater system
Day 3: Majority of larvae set
Day 5: Mass mortality

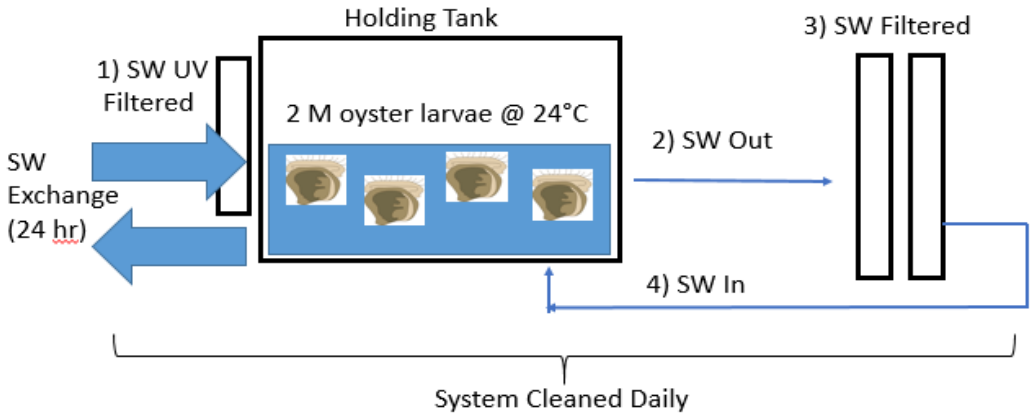
Results: Lab diagnostics (Aquatechnics) report indicated that the presence of *Vibrio* related bacterial lesions were absent, but that digestive gland condition was low and invasive protozoa (ciliates) were present. Cause of low digestive gland condition was food amount and quality, and not bacterial related.

Conclusion: Though recirculating systems avoid drawing excess pathogens from the seawater like standard flow through systems, once an infestation is present, it spreads quickly throughout the system and becomes hard to control. As such, recirculation is not the sole solution to preventing the spread of pathogens in larvae rearing tanks. Additionally, the larvae were underfed throughout the experiment, indicating that food supply should be enhanced.

Experiment 2: The Effect of UV Sanitation on Pathogen Contamination

Objective: Pathogens, such as bacteria and ciliates, can cause significant distress and mortality in hatchery systems, most commonly during summer months. This experiment addresses the ability of UV sanitation to control the levels of pathogens in the system.

Design: Two million eyed larvae were put into a partially recirculating seawater system at 24°C for 11 days starting July 26, 2017. Larvae were fed (at higher levels than in Experiment 1) and seawater was exchanged in the system every 24 hours. The system was manually sanitized daily. Larvae condition and pathogen levels were assessed at the end of the 11 days.



Day 1: 2 M eyed larvae introduced to recirculating SW system
Day 3: Majority of larvae set
Day 4: No signs of ciliates or other pathogen infection
Day 5-6: oysters overfed; system dirty from excess food
Day 7: Small amount of ciliates present
Day 9: 30% infected with ciliates.
Day 11: System was shut down, slow and steady spread of ciliates

Results: Lab diagnostics (Aquatechnics) reported that the larvae were underfed throughout the process. It is possible the wrong combination of algae was used for the oysters, though algal strains were typical of most hatcheries. Ciliates may have resulted in overall mortality, but no other lesions were found and many of the animals appeared healthy despite lack of nutrition.

Conclusion: This report indicates that UV treatment may be a tool for keeping ciliates and bacterial counts low but cannot be depended upon exclusively. It appears that manually sanitizing the tank also could have negatively impacted pathogen populations. Future investigations should experiment with rearing oyster larvae in colder months using typical methods. Many west coast producers agree that growing oyster larvae in the summer is difficult and/or impossible.

In addition to investigating sanitation and pathogen control, PSI provided two real-time multimeter probes for use in the Legoe Bay Shellfish facility. Throughout the project the probes (measuring temperature, pH, DO and salinity) were serviced and calibrated monthly by PSI Sr. biologist Andy Suhrbier. Water chemistry samples were also collected in association with the calibration to gather carbon chemistry values (an important factor to the survival of sensitive shellfish larvae and seed). Prior to the real-time monitoring equipment, Leah and staff used a YSI pH pen, which would often fail or provide inconsistent measurements due to calibration issues. Additionally, pre-existing data probes in the region's Northwest Association of Networked Ocean Observing Systems (NANOOS) monitoring network, of which PSI is a partner, are located in sheltered bays, and don't accurately reflect the dynamic environment of Rosario Strait.

NANOOS data is used often by hatcheries to access local, dependable water quality data, but this data did not exist for Legoe Bay. According to Leah, the reliable data from these new PSI-installed instruments has been essential to understanding the water quality parameters in her hatchery. Results have led Leah to add dissolved sodium carbonate to the water to buffer low pH conditions when they occur. The water quality equipment deployed during this project is, and will be, available to Legoe Bay Shellfish indefinitely, which will allow the small company access to quality data, such as that displayed in Figure 7.

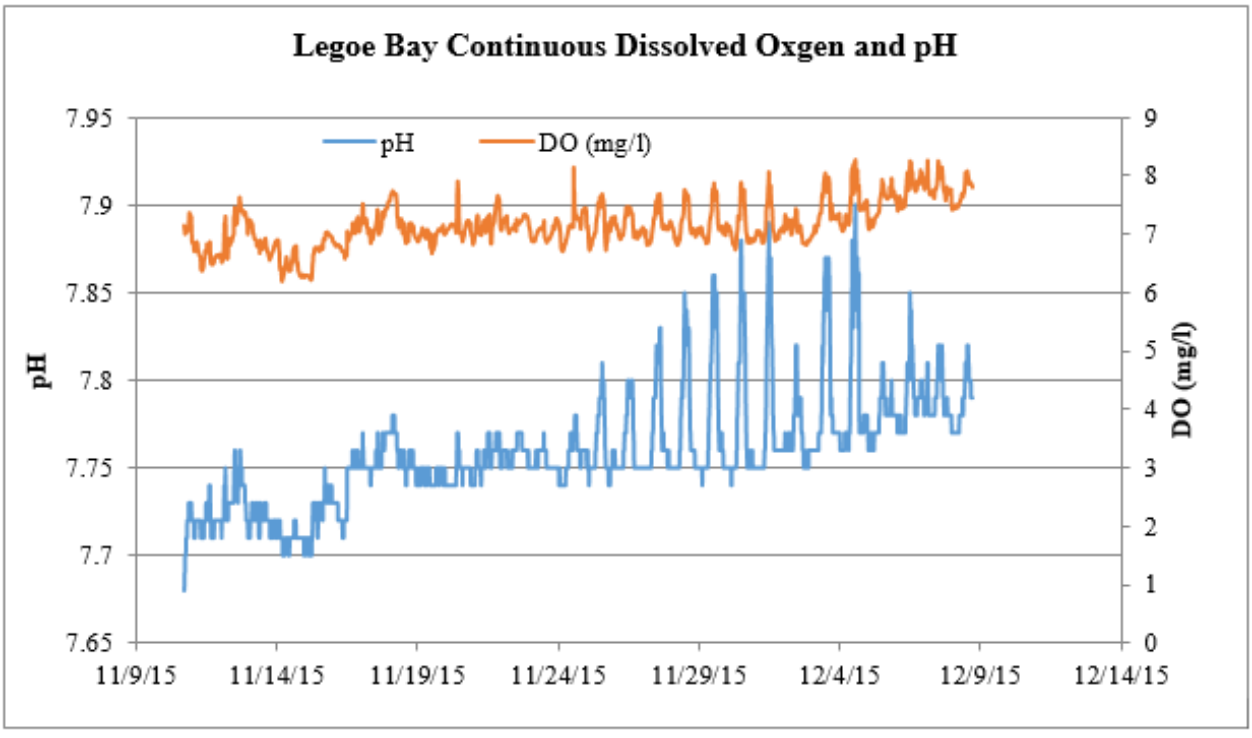


Fig 7. Dissolved oxygen (DO) (right axis, orange) and pH data (left axis, blue) from November-December 2015 at Lego Bay Shellfish Hatchery. Data reflects daily fluctuations in pH and DO.

Entrepreneurs in the shellfish hatchery industry, like Leah, face many challenges. However, forming partnerships with organizations like PSI and Aquatechnics, allows these operations access to consistent data and diagnostics. At the Legoe Bay hatchery, Leah still faces many of the same pathogen issues, but monitors for Vibrio weekly using a PSI-provided incubator for cell culture, and keeps up mechanical sanitation methods to combat ciliate and bacterial contamination. The hatchery has also reduced nutrient loads for algal production, which has decreased bacterial contamination in the juvenile oyster food (a very common problem across the region, as cited by Aquatechnics). Leah is looking forward to expanding on the work that has taken place with water quality and pathogens, hoping to get a better handle of the specific effect each pathogen might have on a system, and also how long-term changing oceanic conditions will impact her facility.

Starting a shellfish hatchery in the remote Pacific Northwest takes perseverance, adaptability, and courage. PSI is grateful to partner with small producers like Legoe Bay Shellfish Hatchery. It is through projects like this, and people like Leah Paisano, that PSI is able to strengthen its connection to the shellfish community and to better understand the spectrum of issues facing producers. Though the challenges of rearing oyster seed at the edge of dynamic natural environments will never cease, the strong network of producers, growers, and researchers will enable the industry to navigate the future challenges to production with greater ease.

Services and Assistance Summary

Understanding water quality data is essential to the success of hatcheries and nurseries. Water chemistry can depend on tidal, diel, and seasonal patterns, and is strongly influenced by the weather. For hatcheries, correctly timing the draw of water into facilities will ensure juvenile oysters have enough oxygen, dissolved calcium, thermal energy, etc. When water quality conditions are poor, hatcheries may adapt by heating water or providing buffering solutions to ensure the proper chemistry conditions (e.g. carbon chemistry) for the larvae or spat to thrive.

Currently, the NANOOS online network of monitoring equipment (www.nanoos.org), which PSI is a member and provider of real-time and cataloged data, can be accessed by seed producers and farmers to view ocean chemistry near their facilities (Fig 8). However, some of these sites have a limited number of parameters and only display data seasonally. Conditions also vary greatly from local influences, and some farms are too remote to depend on readings from these sensors, or have vastly different local conditions (e.g. river inputs, anthropogenic disturbances, etc.).

PSI staff served the seed producers who had additional water quality monitoring needs via the two deployable mulitmeter probes measuring depth, temperature, pH, DO, Chl and salinity on a monthly rotational basis at hatcheries and nurseries in Washington State. The probes rotated between six facilities in Willapa Bay, Totten Inlet, Hood Canal, Dabob Bay, and Harstine Island (Fig 7). After each deployment the data was downloaded and imported to Excel for graphical

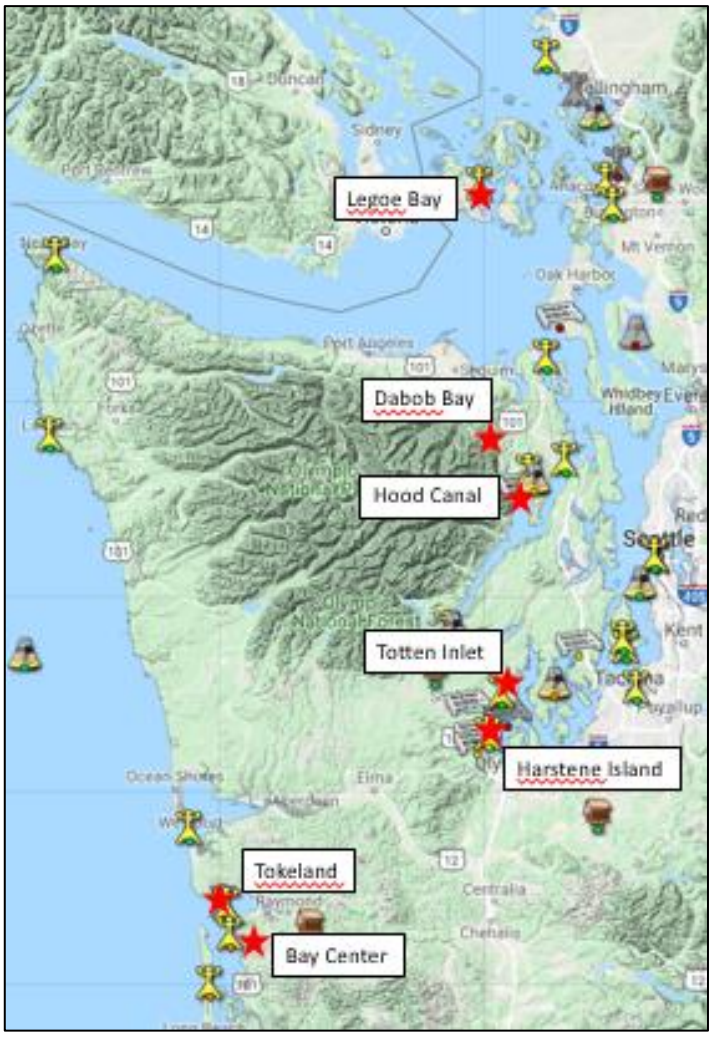


Fig 7. Locations of data buoys (yellow icon) and locations of seed facilities (as represented by red stars) which received water quality monitoring assistance over the project period.

interpretation and provided the facility operator or scientist with notes on relevant data. Meters were cleaned, to prevent invasive species transfer, recalibrated and deployed to another site. Water chemistry samples were also collected in association with the deployments to gather pH and other carbon chemistry values. The data proved valuable to each producer as it enhanced their understanding of local water quality dynamics and have proved to be useful in enabling hatchery staff to buffer and enhance sea water accordingly.

PSI also offered assistance to hatcheries to screen for problems related to plankton, which has been observationally linked to hatchery mortalities. PSI staff screened water samples at a hatchery on Hood Canal, WA, and identified bacteria and ciliate proliferation/contamination in the hatchery system. In this instance PSI, and Aquatechnics provided a probiotic treatment to resolve the pathogen issue . The hatchery found the treatment successful, and continues to use the probiotics as standard practice to bolster the health of the animals.

Addressing Seed Health and Evolving Industry Needs

Shifting Focus: responding to wide-spread adult mortality events

One of the unexpected, yet relevant directions of this project was the high demand for disease testing in moribund adult oysters. There was an overwhelming response by industry managers to utilize seed health funding to obtain health diagnostic data during summer adult mortalities in 2018-2019. Significant mortalities of Pacific oysters have occurred during the summer months in many areas of western North America over the last sixty years, and currently are on the rise in Washington State. On the U.S. west coast mortalities typically begin to occur in diploid and triploid oysters during the second year of growout, often when the oysters are nearing market size. These mortalities have a significant economic impact on individual aquaculture producers.

Fig 8. Pacific oysters (age = 1.5 years) as examined by PSI for an oyster farm in Willapa Bay where mortality exceeded 80%. Pathology suggested particulate irritation in gills, possibly caused by bacteria or phytoplankton

Large-scale mortality events affected growers in all of Washington's major water bodies during the summers of 2018-2019. One producer in Willapa Bay, Washington reported 20-80% loss of near market-ready product over several growing areas during summer 2018. Adult mortalities have proven to be a significant issue for growers, generally from the months of May-August.

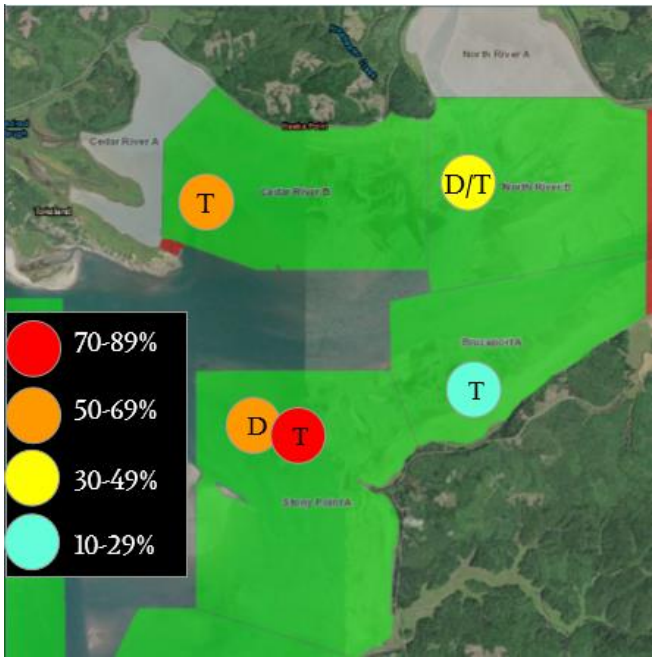


Fig 9. Growing area map from Willapa Bay summarizing the results from a mortality survey done in June 2018. Percent mortalities range from 10-89% and occur in both diploid (D) and triploid (T) oysters. The affected area exceeds 100 acres.

A 2019 Washington Sea Grant Conference for Shellfish Growers session focused exclusively on summer mortality, and attempted to elucidate the causes behind the mortality events. In some cases, adult mortalities could be explained by environmental factors. For example, it has been suggested that a particulate, possibly a pathogen or algae, contributed to the recent mass mortalities**.** However, it is often suspected that a combination of extrinsic and intrinsic stressors (Tan and Ransangan, 2019) contribute to mortality events.

Adult mortality events were reported to, and examined by, PSI and partners during the project period. To best assist oyster growers, PSI conducted mortality surveys on farms and provided the data back to managers or scientists of the percent of product lost on the surveyed parcels. On some parcels, losses exceeded 80%. During the mortalities surveys PSI staff collected and preserved moribund oysters for diagnostic testing at Aquatechnics. When necessary, PSI also provided of the pathology report interpretations to managers, including suggestions for follow up actions. In total, PSI facilitated diagnostic exams for adult Pacific oysters from six companies, and responded to numerous inquiries relating to the 2018 summer mortality phenomenon.

Through this project, PSI facilitated expansion of the Sound Toxins and the Olympic Regional Harmful Algal Bloom (ORHAB) monitoring networks in Willapa Bay to include two underserved areas essential to Pacific oyster production (Bay Center and Nahcotta). This expansion was conducted in partnership with WSG and Washington Department of Fish and Wildlife (WDFW), in response to industry interest. Sound Toxins and ORHAB have traditionally focused on characterizing the three main harmful algal bloom (HAB) species known to impact human health. However, in light of the recent severe mortality events, the focus has expanded to include two plankton species suspected to cause illness and morbidity in shellfish: *Akashiwo sanguinea* and *Protoceratium reticulatum* (Fig 10). PSI has served the Bay Center site weekly from April 2019 to present. To date, a bloom of *Akashiwo sanguinea* has been identified, consistent with WDFW findings from a pre-established monitoring site in Tokeland (7 mi NW of Bay Center, WA). Currently, there is an industry-wide demand for more research on the link between toxic plankton and oyster mortality.

Fig 10. Images of *Akashiwo sanguinea* (L) and *Protoceratium reticulatum* (R). These two species are currently under surveillance by PSI staff at the Bay Center monitoring site, established in 2019. Monitoring plankton toxic to shellfish is part of a larger state-wide effort to quantify these species in relation to mortality events.

Diagnostics and Conclusions from Pacific Oyster Pathology

Aquatechnics Inc. of Sequim, Washington is a shellfish disease testing service for west coast producers. For this project services were provided to assist the Pacific oyster seed production industry to improve the health, productivity and efficiency of seed production. To achieve these goals, the following specific services were provided: (1) Samples of oysters undergoing morbidity or mortality events were processed upon request using histology, (2) Samples of water, algal food production and animal tissue were screened for shellfish pathogenic bacteria using bacteriological methods, (3) Samples were screened for the serious oyster herpes virus pathogen, OsHV-1 var, using a polymerase chain reaction (qPCR) method and (4) assistance was provided using a proprietary shellfish hatchery/nursery probiotic.

Shellfish production companies assisted for pathogen and pathology analysis

The following table shows the type and number of assistance testing by category for shellfish industry partners as a result of this project.

Table 2. Bacteriology samples were from bivalve nurseries and hatcheries. Histology samples -1 represents the number of sample groups of Pacific oyster juveniles and larvae. Each sample group contained multiple individuals. Histology samples -2 indicates the total numbers of individual adult Pacific oyster examined by histology and was used where the condition of such adult samples was believed to directly affect the health of juvenile Pacific oysters. qPCR - OsHV-1 samples were juvenile Pacific oysters, except in the case of adult native oysters and feral adult Pacific oysters from San Diego Bay and were tested to define the risk of the west coast oyster industry to the known virulent strain of OsHV-1 discovered in San Diego Harbor in 2018, during this project.

Company receiving assistance	Bacteriology - water, algae, animal tissue	Histology samples -1	Histology samples -2	qPCR - OsHV-1
Carlsbad AquaFarms				
Chelsea Sea Farms			1	1
Clam Fresh Enterprises	6	9		
Goose Pt Shellfish/Hawaiian Shellfish	2		2	1
Hama Hama Shellfish			39	
Hawaiian Shellfish			67	
Hog Island Oyster Company				4
Jamestown-Pt. Whitney Shellfish		1		
Legoe Bay Shellfish		30		
Pacific Hybreed	1	11		
Port of San Diego FLUPSY facility				5
Rock Point Oyster Company	12	2		
Taylor Shellfish	12	21		
Washington State Shellfish			6	
Feral & native oysters, sentinel oysters, Southern California coast				4
Column totals:	*33*	*74*	*115*	*15*

Histological and bacteriological screening of Pacific oyster seed

Histological examination of oyster seed showed that the following specific causes of lost production were the most common:

1. Inadequate feeding
2. Feed contamination with ciliates or shellfish pathogenic bacteria
3. Invasive ciliates
4. Vibriosis or similar infections (but markedly reduced from prior years)

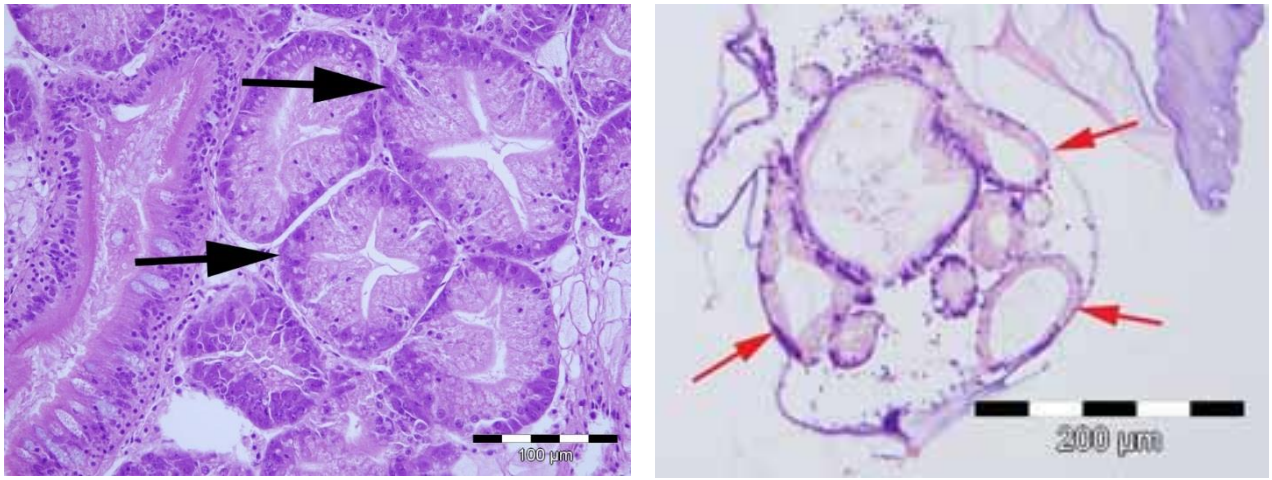


Fig 11. Left hand figure shows healthy digestive gland tubules (black arrows) in a Pacific oyster seed. The right hand figure shows stressed digestive gland tubules (red arrows) in a poorly fed group of Pacific oyster seed. Histology sections using hematoxylin and eosin (H&E) stain.

The consequences of inadequate feeding are slower growth and development and higher risk of predation and disease effects, resulting in lower survival when outplanted.

Invasive ciliates are an intermittent problem in Pacific oyster seed culture. These are clearly distinct and different from typical free-living ciliates commonly observed in cultures. Differentiation and identification of the true invasive ciliate usually requires assistance provided to hatchery and nursery operators. As previously described, the pathogenic ciliates are truly invasive into the body cavity of shellfish seed. The invasive ciliates are slow moving and found in the mantle and body cavity of shellfish seed.

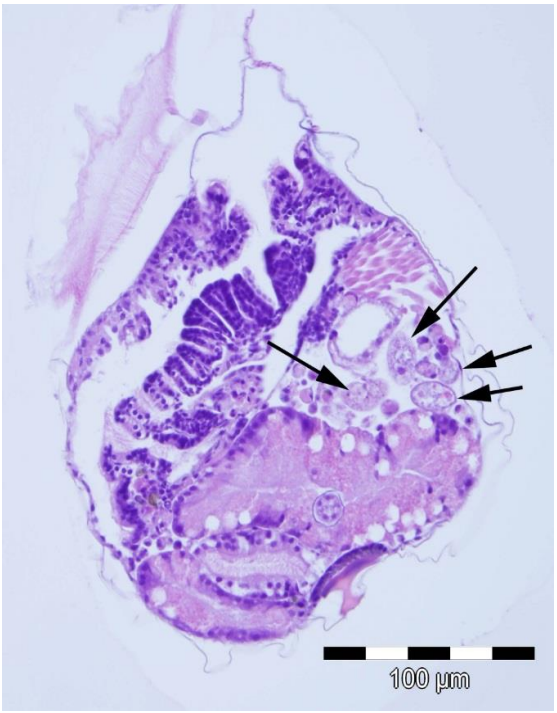


Fig 12. shows invasive ciliates (black arrows) within the body cavity of a Pacific oyster seed. Histology cross section, H&E stain.

Probiotic

Probiotic PO2-45, developed independently of this project was provided to selected shellfish production companies. The probiotic was reported to be highly effective in small hatchery/nurseries systems and reduced mortality losses, while improving the efficiency of algal food production. For this project, probiotic was provided to Rock Point Oyster Company for use between June 2018 and June 2019, providing 50% assistance for payment of the annual licensing fee for the patented probiotic. Rock Point Oyster Company reported good results for the June 2018 to June 2019 year and adopted full payment by the Company for the licensing fee for the probiotic starting in June 2019.

Thus, the project was successful in introducing a useful production aid to a small company which otherwise may not have adopted the cost and assistance provided by the probiotic.

Screening for OsHV-1 virus

During this project, OsHV-1 was discovered, independent of the project, from a sample in San Diego Bay, California, in October 2018. This alarming finding caused concern in the shellfish industry. Thus, in consultation with industry partners, PSI project managers partially reallocated funds for histological pathology to qPCR (quantitative polymerase chain reaction) testing for the presence of the OsHV-1 oyster disease agents. The project was able to assist in defining the risk level of the original finding by screening various samples for this serious oyster disease agent. OsHV-1 microvar had been reported to cause serious oyster death losses in Europe, East Asia and Australia since 2008. Fortunately, in screening 15 separate samples of oysters collected from Southern California sites to Washington State sites, the project found no other examples of OsHV-1 on the west coast of N. America using the qPCR method.

Summary

The following disease or adverse oyster conditions were identified or ruled out as a result of this project:

Inadequate feeding causes Pacific oyster seed morbidity and mortality. Inadequate feeding in nursery was the most commonly found cause of juvenile (seed) losses or poor performance in bivalve nurseries participating in this project.

Invasive ciliates in bivalve nurseries. Invasive ciliates, previously described in the literature, were found to be a severe problem in nurseries, particularly during the spring season.

Vibriosis in bivalve nurseries. Bacterial infections, caused by shellfish pathogenic vibrios, typically *Vibrio corrallyticus* (=*V. tubiashi*) were found occasionally during the study but were far less common that in previous years.

Triploid oyster mortality. Triploid Pacific oysters were found to be more likely affected in mortality and morbidity episodes than diploid Pacific oysters. Triploid sensitivity has been previously observed but the cause is unknown and is a high priority for further research and definition.

Summer mortality. Mortality of Pacific oysters during summer months does not appear to be caused by any specific disease agent, with a few exceptions. This study ruled out oyster herpes virus (OsHV-1) as a cause of summer mortality in all samples of Pacific oyster examined (total of 15 samples examined for OsHV-1 by qPCR). A similar phenomenon with Eastern oysters (*C. virginica*) apparently occurs on the East coast of the United States. In Pacific oysters on the west coast, nocardiosis, a bacterial infection, is responsible for occasional morbidity and mortality of this oyster during summer months but is usually absent in oysters examined for summer mortality. Thus, this bacterial infection is considered a minor cause of the summer mortality syndrome. Summer mortality has been a recurrent problem for the Pacific coast industry

producing Pacific oysters. The cause may be related to abnormal gamete production but a detailed understanding of the cause of summer losses in Pacific oysters is lacking. Thus, more detailed determination of the cause(s) of Pacific oyster summer mortality is a top priority for further research. “Summer mortality” remains largely unresolved.

Oyster herpes virus ruled out in samples tested for this project. Oyster herpes virus (OsHV-1) was very well tested as a result of efforts from this project and the results generated by this project provide confidence that the variant strain of OsHV-1 has not spread outside the original detection location of San Diego Bay, as found in October 2018.

Research Conducted on Remote Setting and Post-larval Seed Culture Methods

Design and construction of a novel, combined setting and primary nursery system for Pacific oysters and importance of upwells for flow through setting

During the course of this project, Baywater/Pacific Hybreed initiated research on the design, construction and resting of a novel combined, setting and primary nursery system potential use by the Pacific coast shellfish industry. As described previously in this report, the industry sector focused on the production of Pacific oysters has seen a decline in oyster seed availability that is due in part to losses at the early post settlement phase of production.

Background on the use of downwell/upwell technologies for setting and growing oyster seed

A number of different setting systems are in use today that, depending on conditions of the quality of the food supply, conditions associated with flow rate, temperature and larval health may work reasonably well for this purpose. A typical downwelling system consists of round silos constructed of plastic or fiberglass pipe sections of various diameter with a screen tightly fit to the bottom of the silo (Fig 13). Seawater is typically pumped into the setting system at the top of the silo with seawater exiting the bottom of the screen (silo on the right), while upwell mode is depicted in the silo on the left side of the figure, with seawater entering the bottom of the screen and exiting near the top of the cylinder. There are many varieties of downwell/upwellers in use by the shellfish industry, ranging from small hatchery-based systems to large FLoating UPwelling SYstems, referred to as FLUPSY’s. The majority of larger systems, including shore and marina based FLYPSY’s are intended for rearing larger seed. For this nursery phase, these systems work very efficiently.

Downwell-based systems are also used for setting and early rearing of oysters in many hatchery locations. The silo in downwelling mode can accommodate small seed placed onto the surface and retained by the screen while seawater (with added microalgae) is delivered to the shellfish via the flow through the silo. Waste materials and any uneaten algae are intended to pass through the screen in the downward flow of water and be removed. As oyster seed grow, the porosity of the screen is increased to accommodate increased flow through the system. When the seed attain a larger size, the system is switched over to upwelling mode. In this case, the flow comes up through the bottom of the screen, passes through the bed of seed delivering both feed and removing wastes in the flow up and out of the silo. The transition from downwelling to upwelling mode is dictated by the size of the oyster seed; setters and small seed are better retained on downwelling mode prior to transitioning to upwelling mode as the seed gain size and weight. The size for the transition from downwell to upwell mode for Pacific oysters is usually at a point when oysters attain a shell length of 1600 microns.

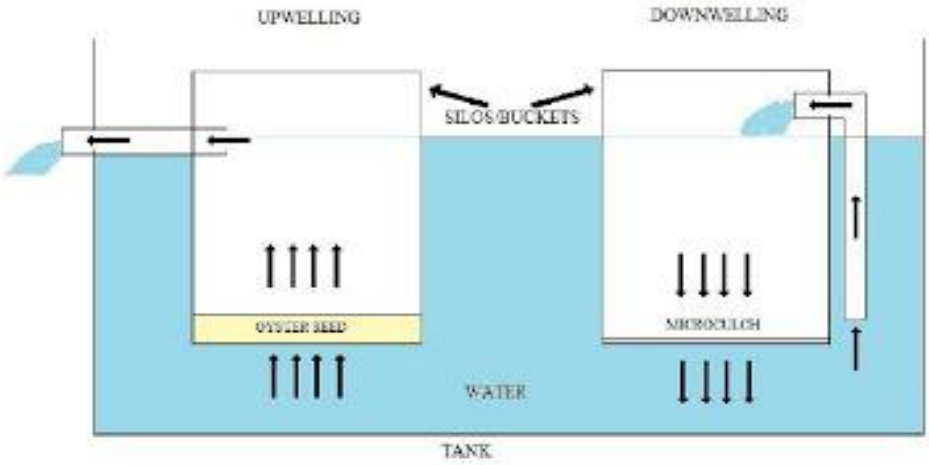


Fig 13. Diagram of simple upwelling and downwelling technology. There is a large variety of systems in use today that vary in size and capacity. All use the same principles of operation.

Fig 14. A large shore-based FLUPSY system located in WA State consisting separate rafts; each raft is powered by a paddlewheel that generates an upwelling current through the base of large bins holding shellfish seed (photo used with permission by Taylor Shellfish Company).

Setting Pacific oysters in downwellers

Typically, downwell silos, if they are used in the setting process for Pacific oysters, are fit with a mesh having a mesh diameter of 180 microns, a porosity that enables pediveligers to be easily retained on the mesh surface while enabling a reasonable flow through the mesh. Once oysters are set and initiate growth of the (calcite based) dissoconch shell, the mesh of the silos is usually increased, first to 400-500 microns and then perhaps to a dimeter of 1000 microns or more to accommodate the larger sized seed. Increasing the mesh in downwelling silos significantly reduces daily maintenance requirements associated with cleaning and rinsing silos as described earlier, attention to the physiological status of pediveliger oyster larvae is critical for successful settlement and metamorphosis of oysters to the seed stage. Pediveligers that are competent to settle and metamorphose to the juvenile stage share these features and timeline to develop to the juvenile stage (at a seawater temperature of 20-25 °C.):

Fig 15. A hatchery-based multi-silo system used for setting and rearing oysters.

- The larvae has approached a shell length of 275 microns (holding on a 245-micron screen).
- The stomach of the larvae is brown in color indicative of being well fed.
- Small drops of oil (lipid) dispersed in the tissues of the larvae and may be discerned through the semi-transparent valve (aragonite based).
- The eye spot in the pediveliger assumes a diameter 10-14 microns.
- Larvae competent to settle tend to "swarm" and congregate in the water column. These may be characterized a long larval trails maintained by mucus threads secreted by the larvae.
- Larvae swim gregariously in agglutinated spiral columns just prior to settlement.
- Larvae typically swim to the top of the column near the water's surface and then cease swimming and fall back to the bottom before rejoining the spiral column.
- On the bottom and in contact with a substrate, the competent larva will crawl and/or rock from side to side on the substrate (often observed on a glass microscope slide) with the foot extended.
- Careful observation will discern that the larvae just prior to settlement turns over onto its left side and attaches to the substrate (utilizing a byssal cement).
- Once cemented to a substrate the pediveliger ceases to feed and initiates metamorphosis to the juvenile stage (24-48 hours).

The flow of seawater through the screen of a silo, whether on downwell or upwell mode, is a prime consideration and control point. Often, hatchery operators underestimate the necessity to maintain an adequate rate of flow supplied to oysters in an effort to conserve heated seawater, microalgae supplied to the system or both. Placing the system on partial recirculation mode will assist I the conservation of both heat and food and can partially alleviate issues associated with the rate of delivery of microalgae to the oysters as well as the rate of waste removal. If the flows

over and through the screens are set too low then the screens are quickly fouled with excess algae and debris and increases the opportunity for pathogenic bacteria colonize.

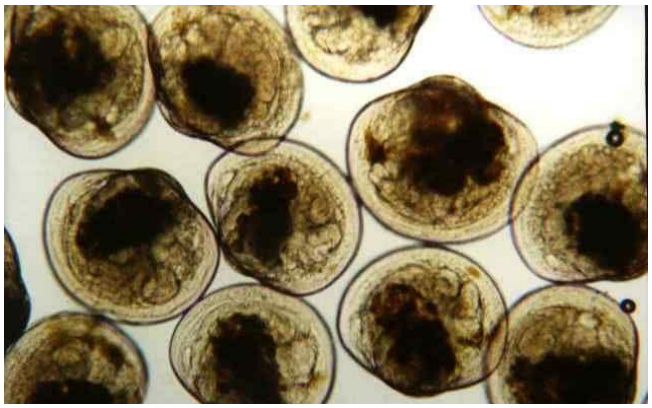


Fig 16. Pre-competent Pacific oyster larvae. Note there is no presence of eye spots.

A direct comparison of two styles of downwell systems operated side by side, with the only difference in the systems being the diameter of the silos and flow rate, offers an example of the importance of flow rate in influencing settlement rate and land early survivorship of oyster seed. This unpublished work was accomplished over a number of months at the Taylor Shellfish hatchery facility in Quilcene, Washington (Joth Davis, unpublished information) and is reflective of similar experiences at different hatchery facilities in Washington State for a number of hatchery facilities, including the Baywater hatchery in Manchester, Washington (Joth Davis, personal communications)

System 1 typically consists of 6" (15.25) cm diameter silos as part of a multi-silo system set on downwell mode to accommodate a flow 1000 ml seawater per minute (similar to the system depicted in Figure 3). The surface area of screens of these silos is 182 square cm. Mean flow rate is easily calculated at 5.49 cubic cm (the volume of seawater passing through the screen per minute per square cm of screen area).

System 2 typically consists of 20" (51.0cm) diameter silos as part of typical 3-silo system set on downwell mode. In this system, the airlift generated flow through the screen is typically set to a flow rate of 5000 ml seawater per silo per minute. The total surface area of individual screens in this system is 2096 square cm. Mean flow rate through the screen is calculated at 2.38 cubic cm (the volume of seawater passing through the screen per minute per square cm of screen area).

In this example, both setting systems were stocked with Pacific oyster pediveligers from the same larval cohort and the density set to 100 pediveligers per square cm of screen surface area. The smaller screens received about 18,200 and the larger system received about 210,000 pediveligers per silo at the start of the experiment, respectively. Replication of the systems included 28 System 1 style silos and 3 System 2 style silos per run. Maintenance included once daily spray-downs of each screen with a saltwater hose to remove accumulated algae and debris.

The results of this experiment (repeated three times under similar conditions) were clear cut. No surviving seed were observed in any of System 2 silos while mean survivorship in the System 1 silos averaged over 30% % after 7 days averaged over three trials. The only significant difference in system output was related to mean flow through the screens with System 2 flow rates on average less than half (43%) that calculated for System 1 silos.

This work pointed to the importance of seawater throughput to accommodate what may be an intuitive need for satisfactory feed delivery and waste removal. Based on these simple results, the flow rate for System 1 appears adequate to serve this critical need compared the lower throughput in System 2 silos.

Further consideration of the fundamental hydrodynamic and other physical conditions facing a pediveliger when placed onto nytex screens at a density of 100 larvae per square cm, suggests that rethinking the fundamental problem is warranted. Downwelling by definition subjects the pediveliger to an avalanche of feed and debris and potentially an entry point for contamination by pathogenic bacteria, unless the cultures are very well maintained. Within the setting of commercial hatchery operations this is seldom the case and alternatives should be considered. Larval oyster recruitment into natural habitats are characterized by some fundamental differences when compared to the artificial systems in use today for setting and growing oysters. First, the density of setting oysters in nature is likely far less than 100 larvae per square cm that is the industry standard. Reducing the setting density for commercial operations is possible but certainly not a preferred option as hatchery and nursery space is usually limited. Second, the physical location for new spat observed in both nature (and setting tanks for spat on shell production) is usually higher in the water column, away from the benthic boundary and microhabitats characterized by a significant flow of seawater. Perhaps the position of oyster spat, and any behaviors exhibited by pediveligers within the confines of a setting tank can help inform better design criteria for high density setting systems?

Fig 17. Pacific oyster spat on shell indicative of a heavy set. Note the spacing between individual oyster seed and may be mediated by the behavior of setting oysters. Density among oysters in a typical setting system for single oysters is far higher.

Design and construction of a combined setting and primary nursery system

Pacific Hybreed personnel initiated design, construction and testing of a novel, combined setting and primary nursery cylinder for use in setting and rearing Pacific oysters in 2018-2019. Several characteristics of a setting system were considered of primary importance during the design phase:

- The system must accommodate setting of pediveliger larvae through metamorphosis and enable easy maintenance and a high percentage throughput to the juvenile stage (>2000 microns).

- The system must enable both setting and post set nursery operations in an upwell mode only in order to avoid issues associated with downwelling seawater.
- The setting/nursery system must be inexpensive to construct, easy to clean and maintain.
- The setting/nursery system must enable simple operation using parts that are readily available to the hatchery operator.
- The system must integrate well in either indoor or outdoor settings and use pumps that were powered by regular 110V power.
- The setting /nursery system must accommodate the production of a commercial quantity of oyster seed as a solitary unit or otherwise enable multiple units to produce commercial quantities of seed (>1,000,000 seed per cohort in multiple units).

Fig 18. Handling the combined setting/primary nursery system at the Pacific Hybreed hatchery facility. Ease of use and capacity are important attributes of the system.

Description of combined setting and primary nursery system

Fig19. Detail of the coupler screen "sandwich" with newly set oyster seed.

A system based on the use of readily available PVC plastic plumbing parts and clear PVC or acrylic pipe was viewed as the best option to consider. The following parts were procured to build a simple pilot system that incorporated the characteristics required:A section of clear PVC pipe (4" diameter) was cut to a length of 19". Next, a pair of slip x slip couplings (4" diameter) were cut laterally in half on a band saw. A piece of nytex screen (180 micron mesh porosity to accommodate Pacific oyster pediveligers) was then sandwiched between the cut sections of the coupler and the two half sections reattached using polyester resin. Care was made to ensure that no spaces were exposed between the screen and the sides of the coupler. If spaces were observed these were filled with polyester putty to ensure the integrity of the screen PVC coupler bond.

Fig 20. Detail of reducer coupling and valve. Reducer slip fits into the base of the coupler with screen.

The couplers were then installed onto the clear PVC pipe section, top and bottom to provide an enclosed volume of pipe section with screens on both ends (Fig 19). Next, the coupler on the

intake side of the unit was plumbed into a set of reducing fittings to enable a pipe section (3/4" schedule 40 PVC) to connect the 4" diameter coupler at the base of the cylinder to a section of ¾" PVC pipe and valve (Fig 20).

Fig 21. A view of the top of the combined setting/primary nursery cylinder system. Note the exit pipe to a common gutter used to collect outgoing flow. In this view, the couplers are fit with interchangeable 475 micron screen, used for holding one week post set seed. All connections are slip fit and require neither glues nor O-rings to maintain a watertight connection.

The valve at the base of the unit is designed to control the rate of seawater flow up through the cylinder. Seawater passes through the top screen and then out through a hole cut into the upper coupler into a collection gutter (Fig 21). The screen sandwiched between two halves of the coupler at the top of the unit serves to securely enclose the volume (4.2L) of the cylinder between upper and lower 180 micron screens. One of the attributes of this system is that the plumbing parts associated with the cylinder may be joined via simple friction and therefore do not require O-rings or other means to ensure a leak-tight fit. To more easily remove the screens from the tubes, silicone grease around the edges was applied. This also aided in creating a water-tight seal.

This last point is recognized as a major attribute of the system as parts can be readily disassembled for cleaning, etc. Multiple cylinders were constructed for testing as a multi upwell unit (Fig 22). Combined setting/nursery cylinders were established for test purposes at the Pacific Hybreed laboratory in spring 2019. Testing of the system utilizing multiple cohorts of triploid and diploid Pacific oyster larvae ensued. The rationale for using a system that relied on upwelled seawater is dependent on setting oysters as cultchless singles using epinephrine as a means to bypass the settlement and cementation phase. By upwelling in a column of seawater pediveligers that were otherwise undergoing metamorphose to the juvenile stage, it was recognized that no substrates were necessary to add to the setting system, thus eliminating the need for micro-cutch. Also, the elimination of micro-cultch from the system removed a substrate that is often considered a refuge for bacteria in the system, including pathogenic species.

Fig 22. Multiple combined setting/primary nursery cylinders attached to wall in the Pacific Hybreed laboratory facility.

Testing the system focused on evaluating the regulation of water flow through the cylinder and to discern whether pediveliger larvae would thrive. It was determined through trial and error that a flow of 300-500 ml per minute would accommodate pediveligers without significantly impingement on the upper (down-stream) screen. In this scenario, pediveligers rise up into the water column within the cylinder and tumble back to the base screen before being propelled back up again. The clear PVC cylinders, in use for both larval rearing bottles and combined setting/seed rearing cylinders easily enables observations that are necessary to evaluate the flow rates necessary to optimally manage the culture system (Fig 23).

Fig 23. Larval bottle stocked with Pacific oyster larvae, seen well distributed in the water column.

What was necessary next was to determine whether pediveligers would thrive and metamorphose to the juvenile stage and whether this system compares favorably or not to traditional, downwell based setting systems. The question of viability was addressed by assessing the growth and survivorship of pediveligers during process of metamorphosis to the juvenile stage.

Methods

Pacific oyster larvae were spawned from hybrid Pacific oyster broodstocks using standard operating protocols and reared to the pediveliger stage. Competent larvae were collected over a two period with pediveligers stored in the refrigerator until use. Pediveligers, stored in damp paper toweling overnight, were removed from the refrigerator and allowed to come to room temperature (45 minutes on the lab bench). Pediveligers were added to a volume of filtered seawater. Aliquots of larvae, corresponding to three density treatments, were removed from the stock culture of pediveligers. Briefly, the pediveligers in the stock culture (a 10L bucket) were distributed in the water column and replicate volumes of larvae in seawater drawn, corresponding to three density treatments. In this case, treatments corresponding to populations of 200,000, 400,000 and 600,000 larvae each with three replicates per treatment. Pediveligers for each treatment were contained in 1-L tri-pour plastic beakers.

An epinephrine solution was made using epinephrine bitartrate salt, dissolving 2g of the salt into a 10ml volume of de-ionized water. One ml of the stock solution, added to 1000ml filtered seawater, results in an epinephrine concentration of 10^{-4} molar in seawater. The nine replicate liters of seawater holding the different populations of oysters were dosed by adding 1.0 ml epinephrine solution to each culture. Regular mixing of cultures was accomplished by regular plunging. All solutions were maintained in the dark for 90 minutes prior to pouring the volume

through a 240 micron screen and rinsing out the epinephrine solution. Treated larvae were added to three replicate combined setting/nursery cylinder for each of three density treatments.

Care for oysters over the following days consisted of the following:

- Cylinders were taken down every other day for counting and cleaning/maintenance. The cylinders were drained one at a time and the top screen lightly sprayed to move all oysters down the cylinder and onto the bottom screen.
- Top couplers were then removed and the oysters gently sprayed into a counting screen and rinsed with filtered seawater. Cylinders were cleaned with a freshwater/Vortex cleaning solution and rinsed with fresh water.
- Seawater was drained daily from the header tank that provided seawater to all nine cylinders. The head tank was cleaned daily.
- Microalgae was delivered via pump to the head tank, cell density was calculated and also the algal mix, header tank, and outflow were measured for cell density twice a day to ensure the oysters were properly fed.

Counting and measuring seed oysters:

- Oysters were originally counted via volume extrapolation from a known volume and population estimates made daily under the microscope. At this size, oyster seed was also measured for shell height using a Moticam camera attached to the microscope at a standard magnification and the data entered on a spreadsheet program interfaced with the camera on an attached computer. This approach was maintained until oyster seed grew to a size large enough to count and measure individually on a stereo dissecting microscope.
- Once oyster seed attained a shell height of 475mm, population estimates for each replicate were made using a "packed volume" approach Briefly, oyster seed from all treatment replicates were placed into a 2ml graduated cylinder in seawater. Tapping on the sides of the cylinder aided to settle the oyster seed to the base of the cylinder. The volume of oysters in the 2ml cylinder was recorded. A table of packed volume equivalents was then used to estimate the number of seed on a packed volume basis, referring to prior research on the relationship between seed count and packed volume of seed retained on screens of a specific pore size (Burge, Davis and Hedgecock, unpublished method).

Once oysters were counted and measured, they were placed back into the cylinders and the process repeated 24 hours later over the course of the 14 day experiment.

Results and conclusions

Oysters that had been treated with epinephrine immediately metamorphosed and initiated spat growth within each density treatment. Growth was satisfactory and after 7 days, mean size for all treatments was 710 microns (Fig 24). After 14 days the size of oysters in the 200K treatments were significantly larger than either higher density treatments with the mean size of seed at 14 days approaching 1400 microns (Fig 25). Survivorship was post set seed was low across all treatments (Fig 26) and may be attributed to the daily handling associated with measurements. After Day 1, survivorship ranged from 5-20% of initial numbers. Losses may also be attributed to overtreating with epinephrine (1.5 hour exposure). Additional research has since indicated that the epinephrine treatment may be fully effective after only 10 minutes exposure. Under routine hatchery rearing circumstances, young oyster setters would not be handled in this system for at least a week post set, other than conducting daily rinses. Survivorship after 24 hours appeared higher in the low density treatments but decreased overall on ensuing days. The system for both setting and rearing seed oysters appears satisfactory and is being further tested at the present time for use with both Pacific and Kumamoto oysters.

Fig 24. Condition of seed approximately 7 days post set in combined setting/nursery cylinder.

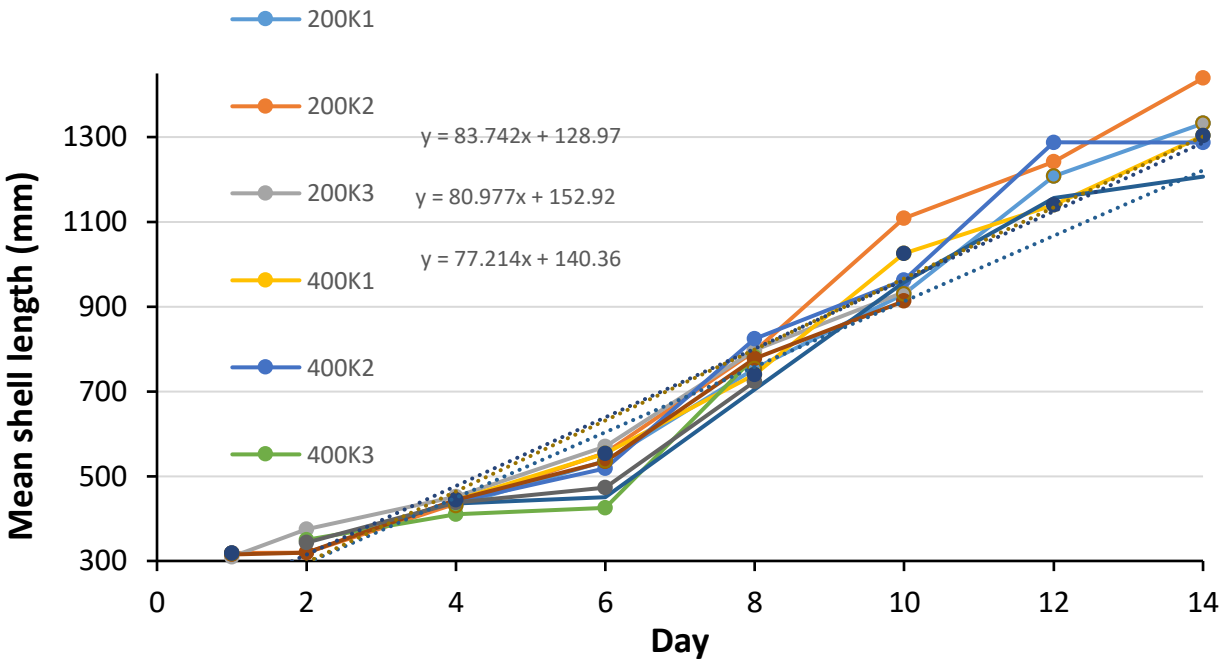


Fig 25. Combined growth for all replicates over the 14 day experimental period. Seed oysters in the lower density treatments were larger than either the intermediate or high density treatments.

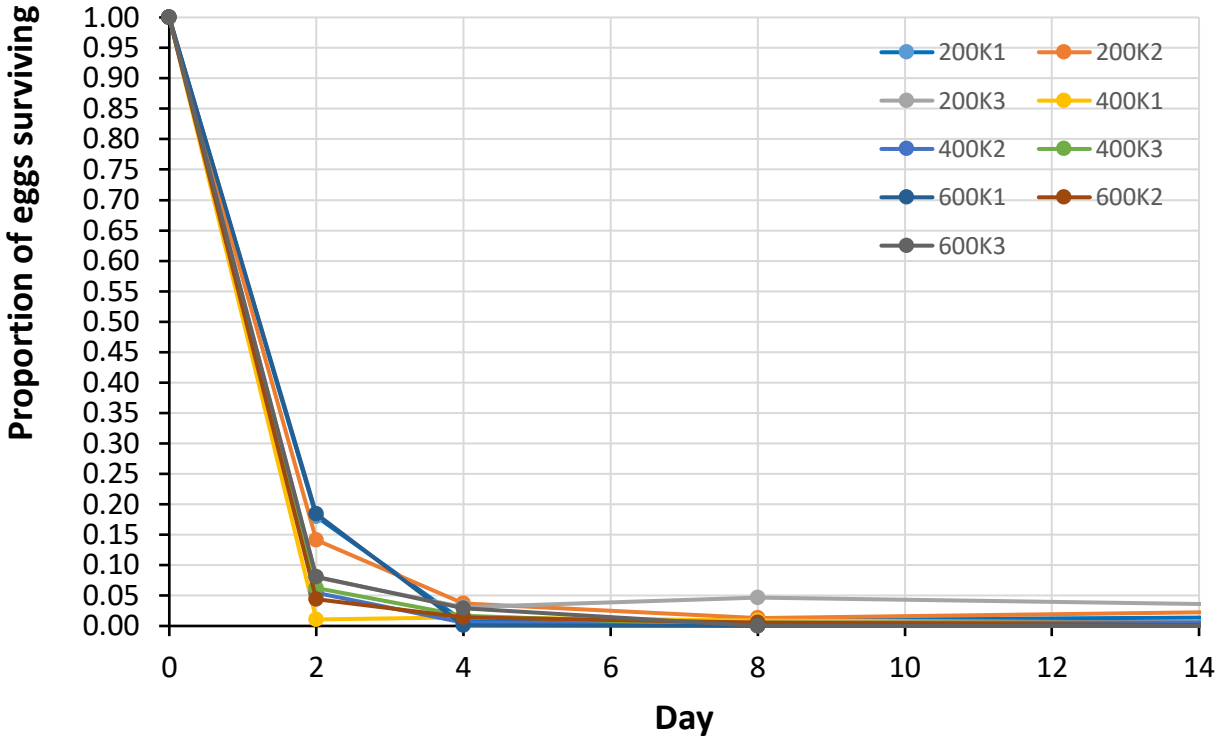
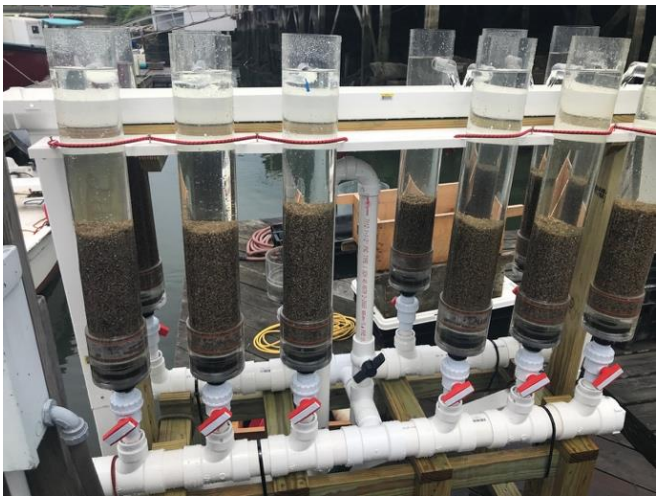


Figure 26. The number of surviving oysters after 14 days was low for all treatments and attributed to intensive handling at the beginning of the experimental period (Day 2).

The Pacific Hybreed research group conducted a series of tests of the use of Reed Mariculture formulated diets to supply nutrition to post set Pacific oysters. A series of test diets were evaluated over the spring and summer 2019. Unfortunately, due to unanticipated decreases in live algal supply and microalgal quality during a critical time frame in summer 2019 needed to conduct final work evaluating the combined setting/nursery cylinders, little comparative data was collected. Preliminary information suggests that the Reed Mariculture Spat diet, soon to be available to the aquaculture industry, holds significant promise as a viable supplement to live algae. More evaluation is necessary however, to validate this statement. The loss of satisfactory volumes of live algae needed to make quantitative comparisons of live algae to Reed Mariculture spat diet during this critical period of evaluation was unfortunate and attributed to quality control issues in the laboratory. These issues have been resolved and ongoing work with the combined setting and nursery systems has continued.

A note on the use of upwelling cylinders for rearing seed is warranted. FLUPSY operators can only accommodate seed that is of a sufficient size to place into outdoor systems. The size class of oyster seed for this part of the operation is dependent on a variety of factors, but often operators of nurseries wait till the seed is at least 2mm before putting into FLUPSY's for boosting the size to 12-18mm for out planting. An intermediate, bottle based system that can be installed at

FLUPSY location may be used to accommodate this size window and enable the operator to move small seed out of the hatchery and into a bottle based system prior to loading into regular FLUPSY bins. Such an operation is in place at a FLUPSY location in Maine and was reviewed during this project to investigate alternate systems that may benefit the Pacific coast oyster industry on the west coast (Fig 26 and 27). The major advantage to this system is the capacity to use natural productivity under largely controlled conditions to boost oyster seed to a size more suitable for shore and sea-based FLUPSY's.



Figures 27 & 28. Outdoor bottle based seed nursery system in Maine. Raw seawater is pumped through the bottle system when natural productivity enables good oyster growth.

Tools developed that aid in quantifying live algal feed for larval and nursery rearing systems

Over the course of this and previous studies it has become apparent that increased scrutiny of the quality and quantity of live microalgae is a critical industry need. Broad experience with hatchery operations has resulted in the general view that most shellfish hatcheries do not use quantitative approaches to measuring feeding rates in shellfish larvae and seed, relying instead on qualitative and empirical observations of performance. While this approach has merit as a timesaving and cost effective component for all of the activities associated with hatchery rearing of larvae and seed, the experience of the industry in recent years with declining success in rearing Pacific oysters for some operators, may warrant more attention be paid to quantitative approaches. Pacific Hybreed has conducted a study assessing the utility of two instrument based approaches to counting microalgae simply and effectively. The first instrument that has been evaluated is the *Luna II Cell Counting Slide*, available from *Logos Biosystems*. This instrument enables the operator to simply and reliably measure the population of microalgae for any number of algal species. The operation of the Counting Cell requires a technician to collect a sample of

algae orlarval rearing water, etc., place a small quantity of sample onto the counting slide and obtain a measure for the population of algae (cells per ml). This instrument is in routine use at Pacific Hybreed's hatchery facility. The second instrument, a hand-held fluorometer (*Turner Designs 2850-000-F Handheld Little Dipper Fluorometer*) enables a simple measure of chlorophyll content in seawater. In this case, the sensor of the fluorometer is simply dipped into a tank of seawater containing microalgae and a measure of chlorophyll content attained. As a possible tool for use by the industry to better evaluate larval and seed nutritional conditions, our laboratory was interested to see if the two instruments could work together. If a technician can use the simple hand held fluorometer to obtain readings for chlorophyll content for the many tanks and cultures around a typical hatchery/nursery facility, could the chlorophyll values taken on the Turner hand held fluorometer be used as a proxy for cell counts made on the Luna II Cell Counting Slide?

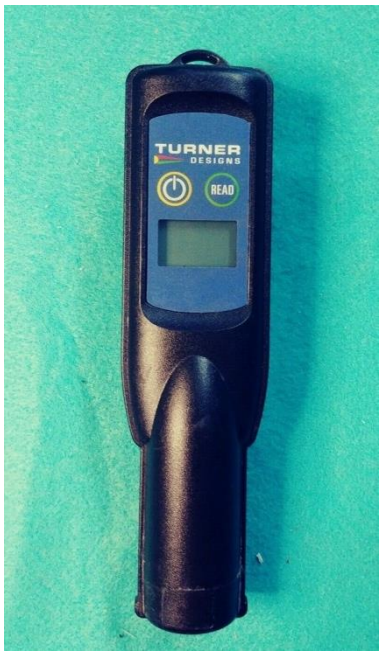


Fig 29. Turner Designs hand-held fluorometer.

A simple study was made to assess the relationship between the chlorophyll content of a routinely cultured algal species and the cell count for the same sample assessed using the Luna II Counting slide. Briefly, serial dilutions were accomplished using the green microalgae, *Tetraselmis suicia*, as a test species. *Tetraselmis* was added to filtered seawater as a percentage of the total volume in a beaker and replicate samples measured using both instruments. Data was taken and plotted, resulting in a good correlation between chlorophyll content and cell number for this species (Fig 29). A simple linear regression then enables to inform the hatchery technician of the algal cell count present in the culture based on the fluorometer reading (Fig 30). In this case the regression generated a highly statistically significant relationship between chlorophyll concentration and algal cell abundance. This approach may assist in enabling better quantification of algal feed rates overall for broodstock, larvae and seed for hatchery operations moving forward. It should be stressed that the operator should assess chlorophyll content for the microalgae species cultured independently of this study as culture conditions will vary from facility to facility and result in potentially different chlorophyll profiles on a species specific basis. The overall approach, however, appears promising.

Fig 30. The Luna II Cell Counting Slide.

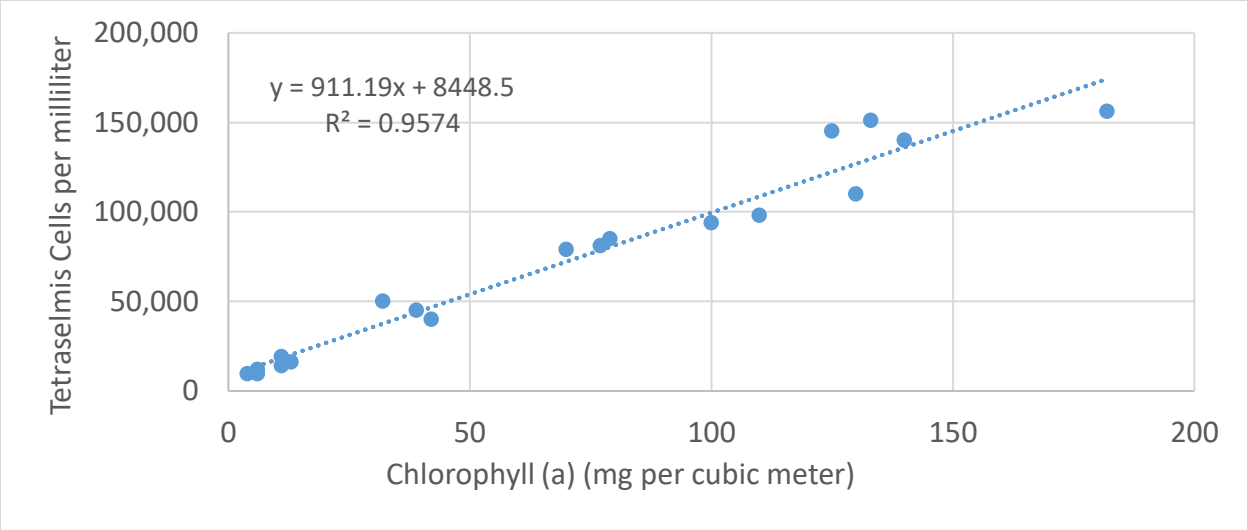


Fig 31. Correlation between chlorophyll a value and algal cell counts made on a hand held fluorometer and a cell counting instrument.

Inventory and evaluation of new and existing systems for remote setting and early post-set seed rearing

Introduction

Remote setting of oyster larvae (also referred to as "spat on shell production") has been an industry practice for cupped oysters within the genus *Crassostrea* for the last 40 years. Techniques for setting Pacific oysters (*Crassostrea gigas*) were pioneered by Lee Hansen, long-time owner and operator of the Whiskey Creek Shellfish Hatchery located on Netarts Bay, Oregon. Early research on methods for remote setting was provided by researchers at Oregon State University's Hatfield Marine Center working with Whiskey Creek in the early 1980's with fundamental information on optimal conditions of temperature and salinity required for satisfactory production established by Henderson (1983). Since that time many refinements have been made to the fundamental approach that have served to optimize production.

The focus of this component of the project was to evaluate current seed rearing practices for the Pacific oyster and propose changes in husbandry that may serve to increase the survivorship of oyster seed to the benefit of the shellfish industry on the west coast. This report focuses on an evaluation of new and existing systems for remote setting and early post-set seed rearing. To that end it should be recognized that to focus on setting and early post set survival of Pacific oysters only was to neglect critical husbandry practices in the production of oyster larvae that may materially benefit post settlement production overall.

It is also important to recognize that for reasons not completely understood that commercial rearing of Pacific oysters today has become more problematic than it was 20-30 years ago. Issues associated with larval mortalities at various points in their larval life history, poor survivorship to the setting stage and poor seed survival have become routine for hatchery operators in the Pacific

Northwest of North America. Since about 2007 oyster production in the Pacific Northwest has been compromised from time to time for nearly all hatchery producers due in part to changes in the quality of seawater suitable for the hatchery culture of oysters impacting the shores and estuaries of North America. Changes in carbonate chemistry of seawater have been recognized as a major issue impacting the capacity to rear oysters since the mid 2000's. Increasingly corrosive seawater having a reduced pH, increased concentration of dissolved CO_2 and reduced aragonite saturation were found to significantly impact oyster larvae at early developmental stages (Waldbusser et al., 2011; 2013, 2015). While workarounds have been developed that mainly focus on providing seawater to oyster larvae and young seed that has been buffered by addition of sodium carbonate to increase aragonite saturation state, there are other, more poorly researched factors in play including bacterial toxins (proteases) associated with *Vibrio* spp. and poorly understood impacts associated with toxic metabolites associated with native algae, pollutants including hormones, heavy metals and other contaminants that may influence the capacity for routine production of oysters. The problem extends to oyster production around the world. Recent news of large-scale mortalities of oyster larvae in southern Korea, for example has brought the hatchery industry there for Pacific oysters to a standstill (Eric Henry, personal communication, 2019). The bottom line is that much remains to be learned as the production landscape for Pacific oysters appears to be changing along with the environment in an era of rapid climate change.

What also appears important to recognize is that successful cultivation of Pacific oysters does not begin with receipt of generic pediveliger oysters from the hatchery for either remote setting. Rather, attention to the nutrition of broodstock that subsequently produce gametes with optimal biochemical reserves is a prerequisite along with attention paid to the nutrition of larvae to optimally produce pediveligers competent to settle and metamorphose to the juvenile stage.

This report may be considered as the basis for developing a revised manual for the production of Pacific oysters that takes into account a better understanding of the fundamental physiology of this species of oyster. A review of the available literature has revealed that hatchery practices in common use today need significant modification to accommodate advances in understanding in order to make practical advances that benefit the industry. An evaluation of oyster production techniques that focus on the hatchery production of oysters from broodstock management to remote setting techniques that can provide enhanced production of seed may provide insights into how techniques in use today may well be optimized. What follows is an assessment of protocols in use by the industry today for production of the Pacific oyster from egg to the seed stage. The hope is that insights provided here can assist in boosting production of oyster seed, in line with this project's objectives. Identifying factors based on the physiology of the oyster as well as published literature that has been either ignored or forgotten by the industry that can increase the efficiency of Pacific oyster production is the primary focus of this report.

The physiology of Pacific oysters associated with optimizing broodstock conditioning

There is increasing evidence that larval survivorship beyond the straight hinge developmental point may be influenced by specific conditioning protocols used for Pacific oysters. Within the

context of the research reported here, what follows is a critique on past research and practices used by the shellfish industry and preliminary research on broodstock conditioning in Pacific oysters that may assist hatchery operators in producing more and higher quality oyster seed. It is suggested that closer attention be paid to both published research and the specific physiology associated with storage cycles and reproduction in Pacific oysters in order to potentially increase the efficiency and outputs for the oyster industry sector focused on *C. gigas*.

The condition of broodstock oysters in the field and their status relative to storage metabolism is important to assess. Control over this feature is difficult to control as nutrition of oysters in the field is difficult to control but having oysters in good condition relative to stored reserves is very important for later success. The movement of oysters to "fattening ground" or areas that are richer in natural phytoplankton abundance at some times of the year relative to other locations has been a feature that growers have long used to ensure that oysters brought into the hatchery environment for subsequent use as broodstock are in good condition (e.g. high in stored glycogen).

The role of lipid metabolism in the production of both gametes (during the conditioning phase) and during larval feeding phase necessitates that attention be paid to the provision of feeds rich in highly unsaturated lipids. Because reproduction in molluscs is metabolically demanding, it requires that the organism store a significant amount of resources in preparation to initiating gametogenesis. Hatchery operators routinely condition oysters for reproduction outside of the normal window for natural production. As part of the inventory of techniques used for successfully producing Pacific oysters it is important to recognize that a wealth of information suggests that successful production of larvae and seed depends upon adequate nutrition of broodstock oysters when production is attempted outside of the natural cycle of reproduction for this species. Because acquiring nutrients, accommodating the time frame needed for gamete production and spawning are often tightly coupled to both the availability of resources and the suitability of the environment to support successful reproduction. A strong correlation exists between the health, resilience and vigor of marine larvae to the specific biochemical and physiological conditions in the broodstock used for production in hatcheries world-wide. But these three functional activities associated with reproduction can also be out of phase and lead to significant challenges. Hatchery operators do not routinely screen oyster broodstock gametes for lipid content, nor are oyster broodstocks managed to maximize this critical biochemical component.

Practically, however this interplay among the availability of nutritional resources, endogenous cues that serve to initiate reproduction and the successful production and spawning of gametes may be controlled. This has been a minor component of hatchery management for oyster production for many years. That said, it is also commonly observed that fundamental issues arise if this critical interplay between the availability of resources and their allocation triggered by endogenous cues, if ignored can lead to sub-optimal performance in the production of oysters. This is most often seen at the nutritional stage of broodstock care.

Nutrition supplied to oysters held for broodstock and the production of gametes for larval production in particular has received significant attention in the scientific literature over the years. However, very often oysters collected in the field for broodstock use are selected more for convenience than for their qualitative value for gamete production. Oysters coming into the hatchery during the winter months for conditioning are often nutritionally stressed. Unfortunately, fundamental biological considerations related to the specific nutritional requirements of oysters are then downplayed in the effort to produce viable gametes, especially as this relates to lipid requirements necessary for the optimal production of eggs and sperm. A common practice is to collect oysters in the field during the early winter months and introduce them to the hatchery environment. Oysters are typically fed a mix of hatchery cultured algae over a period of weeks at elevated seawater temperature (typically 18- 22°C) to induce gametogenesis. Cultured species used for conditioning Pacific oysters typically include the Prymnesiophytes (*Isocrysis* aff. *galbana*, (clone T-iso; *Tahitian Isocrysis*) and *Pavlova lutheri* (NMFS Milford Shellfish Laboratory clone 459), the diatoms *Chaetoceros calcitrans* and *C. gracilis*, the green algae, *Tetraselmis suecica*. Other commonly cultured algae used for conditioning oysters for out of season gamete development include the cryptomonad, *Rhodomonas salina* among some others. Feeding of broodstock oysters in the hatchery environment is often characterized by significant underfeeding.

A rule of thumb is to feed oysters a mix of live algae totaling at least $2x10^9$ algal cells per oyster per day. For a tank of 100 oysters fed a typical algal culture of live algae having a cell concentration of 2.5x106 cells per ml, this equates to a feed ration of at least 800 ml per oyster per day. For 100 oysters in the tank, this equates to a minimum of 80 L per day supplied to the oysters as a maintenance ration. If oyster broodstocks are maintained on flow through conditions, a component of the algal feed is wasted and should be accommodated by providing excess feed to the tank.

A number of research investigations conducted in the early 1980's (Lannan, 1980a, 1980b Lannan et al., , 1980c; Muranaka and Lannan, 1984) and by Villalba et al.(2002) documented the critical relationships between temperature, supplemental feeding and the rate of gametogenesis in Pacific oysters. Hatchery operators in the Pacific Northwest generally follow the basic parameters outlined in these important papers. Managers generally introduce broodstock into the hatchery for conditioning in late Fall and early winter, depending on the schedule for first spawns of the season. Protocols described in Muranaka and Lannan (1984) for example prescribe an approach based on the use of degree days. Oysters are generally placed into broodstock tanks in either tanks supplied with running seawater or seawater that is changed out every day or so, at the ambient temperature they were experiencing in the field. The temperature is ramped one degree per day until the conditioning temperature is reached (usually between 18-22°C.). Oysters are maintained at the prescribed temperature for the number of days required to produce ripe (viable) gametes, based on a prescribed feeding regime and degree days.

A significant flaw in Muranaka and Lannan's (1984) approach however is evident as their calculations are predicated using a baseline temperature of 0° Celsius, instead of more properly adopting a baseline temperature reflective of the conditions that oysters are typically observed in

the Pacific Northwest, closer to 6-8°C. For example, a degree day calculation of 700 calculated by multiplying a conditioning time frame of five weeks (35 days) by the conditioning temperature of 20°C. to reach a calculated degree day total of 35x20=700 (Muranaka and Lannan (1984). In fact, the calculation should be based on a baseline calculation of 20° less a baseline value (higher than 0) that is reflective of the actual field conditions and ambient temperature the oysters experienced. This modification can result in hatcheries expending significantly more resources than are necessary during the oyster conditioning phase in order to reach the prescribed threshold degree day total of 1100, a value often used in the shellfish industry to base the time of condition to produce viable gametes for Pacific oysters.

The following example illustrates the approach advocated by current practices in use today. Oysters are typically introduced to the hatchery environment after collection from a local bay characterized by ambient conditions of temperature, salinity and seston concentrations of 8°C and 29 PSU and < 0.5 mg per L particulate organic material , respectively. Oysters are placed into tanks supplied a fresh source of seawater maintained at ambient temperature and salinity. The seawater temperature of the tank is typically raised 1 degree per day until a target temperature of 18-22°C is reached. According to current industry practices, degree days would be calculated as follows: for every day in the conditioning tanks that the temperature is being ramped to the 22°C endpoint, degree days are calculated and summed. Over the initial twenty-four hours, the ambient temperature is increased 1°C. from 8-9°C. In this example, a total of 9-degree days would then be achieved (9-0=9). A ramp of 1 degree per day for the following 12 days required to increase the temperature from the holding temperature to 22 degrees therefore represents a total of 185 degree days. According to industry practice, a minimum of 1100 degree days are required to bring at least 50% of eggs in Pacific oysters to a ripe stage. n this example, to reach the 1100-degree day total then, an additional 42 days would be needed to ripen gametes at a conditioning temperature of 22°C. The total conditioning period in this example is therefore 42 + 12 or a total of 54 days. The cost associated with holding oysters over this time frame is therefore significant.

A revised Conditioning Protocol based on an initial thermal shock

At the Baywater, Inc. hatchery, a study was initiated in early 2017 that considered these industry practices that included published parameters but modified the general approach as follows in order to develop a revised Pacific oyster broodstock conditioning protocol. First, if a threshold temperature is applied to degree days calculations, then significantly fewer degree days are actually calculated over a 54-day conditioning window. In this example, a threshold temperature of 8°C. is used as the temperature oysters were observed in the field just prior to introducing to a conditioning window. Degree days are calculated as follows: a ramp of 1°C on the first day from 8°C to 9°C represents only one-degree day. Subsequent days over the temperature ramp are then calculated using 8°C. as the baseline.

In this example, to reach the 22° conditioning threshold over the 12 days required for the ramp from 8-22°C, represented a total of 96 degree days. If 42 additional days are required to condition oysters under normal conditions, only 588 additional degree days are needed, for a

total of 684-degree days. This then represents 62% (588 compared to 1100) of the total suggested degree days typically applied by the industry. Hatchery operators using the 1100-degree days as the threshold necessary for a typical conditioning window may then be consistently expending significantly more time and resources to produce oyster broodstocks. This can also result in oysters being conditioned longer than necessary and be significantly overripe at the time of use.

On the other hand, if the calculation of degree days is made, using the more appropriate "threshold temperature" approach then oysters may be conditioned with better control over the rate of gamete development. This is a problem that has gone largely unrecognized by the industry and results in hatcheries producing overripe gametes while wasting valuable resources and time. Published observations on larval survival and vitality depend upon the optimal utilization of Pacific oysters with a focus on the use of gametes that are undergoing the latter stages of oocyte growth (e.g. vitellogenesis). This is often not the case as hatcheries may use gametes for as long as oyster broodstocks show any presence of gametes, even when oocyte growth has long ceased. This is a very important distinction that hatchery operators need to recognize and potentially incorporate into their oyster conditioning protocols.

To this end, an important paper by Cannuel and Beninger (2005) identifies four critical components or in their word's "predictors" of optimal egg quality in *C. gigas*. These include the stage of gametogenesis, the diameter or volume of the average oyster egg in the gonad, the lipid content of the egg and the presence or absence of gonadal atresia. This last term refers to any indication that eggs are being resorbed and are therefore past their optimal condition. In addition, a number of metrics validate the predictors and include the percent of larvae developing to the straight hinge stage, larval feeding rate, larval growth rate and post larval growth. Added to this list of metrics is the time in hours to the pediveliger stage and the percent of pediveligers that survive to the juvenile stage, often defined as holding on a 2000-micron screen (J. Davis, unpublished observations).

In practical terms, optimal survivorship of Pacific oyster larvae to the seed stage is predicated on using optimally conditioned broodstock with no evidence of gonadal atresia. This is difficult to determine unless histological methods are used, so a general rule is to apply just enough degree days of conditioning to produce oysters capable of spawning.

There is an alternate approach based on original work by Baywater, Inc. based on work during this current project indicating that hatchery resources may be significantly conserved if standard operating procedures are modified to more closely utilize the overall physiological capacity of Pacific oysters to produce gametes over a short period of time.

Experiment 1: Optimizing broodstock conditioning for optimal gamete production

Pacific oysters are often exposed to natural temperature swings of up to 35 °C on tide flats in the Pacific Northwest. Wide variance in thermal exposures may be encountered on intertidal beaches during summer months when oysters are exposed to the air during low tides and then covered by overlying waters as the tide comes in. These conditions may occur at the precise same time when oysters are developing gonadal tissue for later reproduction. Temperature stress within the

physiological range for an organism may also stimulate the transition from storage metabolism to active gametogenesis.

The preliminary hypotheses being tested here is to consider whether Pacific oysters can in fact be "preconditioned" to survive wide swings in ambient temperature under stressful conditions that also serve to trigger rapid gametogenesis and the production of gametes. What follows is a description of the work and results of a preliminary experiment. That focused on alternate approaches to optimal broodstock management. Further assessment of the work is necessary to validate this work. The initial work for this approach was conducted at the Taylor Shellfish hatchery with later work performed at the Baywater Hatchery facility in Manchester, WA under the auspices of the current SK project.

Methods

Oysters from a naturalized population of Pacific oysters were brought into the hatchery from Thorndyke Bay in late winter 2017. Ambient temperature, salinity and seston concentrations at that time were 8°C, 29 PSU and 0.7 mg POM per L, respectively. The visual condition of the meat condition of oysters based on shucking 5 oysters in the field suggested that oysters were in good to excellent physiological condition with tissues filling the internal volume of the animal. There was no evidence of gametogenesis having been initiated in this cohort. Oysters (N=80) were scrubbed free of epibionts and placed initially into shellfish cages maintained at ambient seawater temperature and salinity with no supplemental feeding other than the seston provided in raw seawater for about 5 days.

Conditioning trials consisted of three treatments. In the first treatment, oysters (N=20) were conditioned using the traditional approach of ramping the temperature one degree per day to the target temperature of 22°C. This is referred to as T-traditional. The second treatment consisted of placing oysters (N=20) directly into a tank maintained at the target temperature of 22°C. This treatment is referred to as T22. The third treatment consisted of placing oysters (N=20) from the field directly into a seawater tank maintained at 28°C. and referred to as T28-22. For this group, 28°C was maintained for 4 days and then this cohort was placed into the 22°C. treatment tank for the duration of the experiment. A Control group of oysters (N=20) was maintained at ambient temperature of approximately 8°C. for the duration of the conditioning trials.

All broodstock were fed an algal ration at a feeding level appropriate to the size and number of oysters in the conditioning tank. Because algal feed quality can vary greatly with the culture conditions associated with the production of microalgae, these studies all utilized a Reed Mariculture formulated diet, referred to as Shellfish Diet 1800 (SD1800) for the purpose of feeding broodstock oysters over the conditioning period. Prior experience with SD1800 has demonstrated the efficacy of this product for conditioning oysters in past investigations. Feeding was accomplished as follows. Broodstock tanks were dosed with SD1800 according to manufacturer's recommendations.

Briefly, Shellfish Diet was maintained in the refrigerator at approximately 5 °C. On a daily basis, the bottle was shaken and then allocated to broodstock tanks on the basis that one ml SD equaled

approximately 2x109 cells. The SD1800 was first suspended in a 2-L volume of fresh water to initially disperse the cells. Following initial mixing, SD1800 was allocated to broodstock tanks to ensure a daily ration of $2x10^9$ cells per day was fed to each oyster. For broodstock tanks holding 20 oysters, this amount of SD1800 was 1 ml per oyster per day, or 20ml per broodstock tank, fed out twice daily for a total of 40 ml per tank. Mixing of SD1800 was achieved by placing air stones in individual broodstock tanks to ensure adequate mixing.

The degree day conditioning approach based on using a threshold temperature for calculation of degree days was used in this work to validate the approach for Pacific oysters. Oysters were placed into their individual treatments and maintained for thirty-five days. Sampling of oysters (N=3 per treatment and control groups) for a visual cue of gamete development was used as the primary metric for validation of active gametogenesis. This consisted of taking small samples of gonadal tissue via a glass Pasteur pipette and placing the material on a glass microscope slide and examining the material under various magnification using a stereo compound microscope. Samples were scored for the presence or absence of ripe gametes.

Results

Experimental results for the conditioning trial are summarized in Table 3.

Table 3. Results of conditioning trials for Pacific oysters. Values indicate the percent of male and female oysters that exhibited ripe gonads at weekly intervals over a five-week sampling period. Survivorship values indicate the % surviving oysters after five weeks of exposure.

Treatment	T-traditional	T-22	T-28-22	Control
Oysters (N)	20	20	20	20
W1-male (%)	0	0	0	0
W1-female (%)	0	0	0	0
W2-male (%)	0	0	100	0
W2-female (%)	0	0	100	0
W3-male (%)	0	0	100	0
W3-female (%)	0	0	100	0
W4-male (%)	0	0		0
W4-female (%)	0	0		0
W5-male (%)	0	75		0
W5-female (%)	0	50		0
Survivorship (%)	80	85	30	90

For the T-Traditional cohort, a total of 389-degree days was the calculated exposure. Oysters were subjected to a 14-day temperature ramp to the target temperature of 22°C and maintained for an additional 21 days at that temperature. Oysters were shucked and examined for the presence of gonadal material at weekly intervals and at the conclusion of the conditioning period (35 days total). For each sampling interval, four oysters were shucked and the gametes examined. Mortality in the T-Traditional cohort was 20% (16 of 20 oysters were alive after 35 days).

There were no oysters examined that appeared to have ripe gametes using this approach for any sampling interval. In several cases, there was evidence of male gonadal development, indicating that some ripe spermatocytes were present. There was no other quantification made. In females, egg development appeared to be underway after 35 days but with significant development necessary to achieve the mature stage. No ripe female germinal tissue was observed at the conclusion of the 35-day trial.

For the T22 treatment, a total of 490-degree days were experienced by oyster broodstock. In this case, as described above the oysters were placed from ambient temperature immediately into 22°C. seawater for the duration of the experiment. For this treatment, the additional degree days apparently resulted in the observance of fully ripe male gametes after 28 days and 35 days of exposure. Egg development appeared further along as well but few ripe eggs were observed at 35 days. Survivorship of oysters in the T22 exposure was similar to the T-Traditional treatment. In this case, 17 of 20 oysters were alive at the conclusion of the experiment (85%).

Foe the T28-24 exposure, a significant number of ripe gametes (male and female) were observed after 14 days of exposure following 220-degree days of exposure to elevated temperatures (4 days at 28°C followed by 10 days at 22°C.). In this treatment, in addition to the visual quantification of ripeness, gametes were strip spawned from individual male and female oysters to test whether eggs were viable after such a brief conditioning window. Briefly, gametes from 3 female and 3 male oysters were strip spawned by making small incisions in the gonad using a sharp scalpel to expose gametes. Eggs and sperm were subsequently rinsed into a 100 mL beaker using a squirt bottle for holding. Eggs were incubated for up to 45 minutes in 100 ml beakers (N=5) prior to fertilizing with excised sperm. In all cases, eggs were readily fertilized and initiated development after about 15 minutes. In all cases, initial polar body development was observed. As opposed to the two other treatments, survivorship of oysters past 14 days was significantly lower. Initial mortality occurred after 3 days of exposure to 28 °C after coming from seawater having an ambient temperature of 8 °C. In this treatment, mortality after three days was 40% (12 of 20 oysters were alive after three days). Further mortality occurred in this group, culminating after three weeks at 70% mortality. The trial was terminated after three weeks due to continuing mortality in this group. For the control group no oysters were observed to have progressed in gonad development. Survivorship was 90% (18 of 20 oysters alive after 35 days).

Discussion

Oysters in different treatments behaved very differently based on their exposure history to elevated seawater temperature. Oysters in the traditional treatment were not fully ripe after 35 days of exposure. Oysters subjected to the immediate exposure of the target treatment temperature showed elevated gametogenic development, while oysters exposed to an initial temperature of 28°C (reduced to 24°C after four days) showed significant gametogenic development after only two weeks and gametes stripped from oysters in this treatment developed into what appeared to be normal fertilized embryos. Reliance on degree day determinations may not a reliable approach for conditioning Pacific oysters, based on these results as oysters were

ripe enough to provide fertilizable gametes after just 220 degree days exposure. A significant increase in mortality of oysters exposed to the T28 treatment was significant, however. This resulted in significantly fewer oysters surviving after three weeks of exposure as compared to the other treatments and control groups. Survivorship of broodstock oysters using this approach must be evaluated relative to the benefit of producing oysters provisioned with ripe gametes after only 14 days. If oyster broodstocks are plentiful then the increased mortality using this conditioning approach may warrant its use when ripe oysters are needed to supply hatchery needs.

These results, while preliminary in nature, suggest that endogenous cues associated with stimulating the physiological shift in resource allocation from storage to gametogenesis in Pacific oysters is triggered by a temperature shock. In this work a rapid transition from storage (glycogen mainly) in Pacific oysters was quickly reallocated to germinal tissue production and resulted in viable gametes being produced after just 14 days. Much work remains to refine this approach. Recently proposed work has focused on identifying the precise timing, magnitude and duration of the temperature shock to oysters in that produces rapid gamete production while minimizing adult mortality. Also, the physiological state of the oyster needs to be better defined. If oysters are nearing the conclusion of the annual storage cycle and have adequate supplies of glycogen stored in the adductor muscle, mantle, labial palps, digestive gland and gonadal tissue to rapidly transition to germinal tissue then it's likely that oysters can produce gametes quickly under these circumstances. Otherwise, if oysters are out of condition and lacking in stored reserves then the conditioning regime must also accommodate energy acquisition during the conditioning phase. Oysters appear to have the capacity for both approaches (capital-breeding strategy vs income-breeding strategy) as proposed by Jonsson, (1997), though experience has shown that oysters coming into a conditioning phase produce more and higher quality gametes then when coming into a conditioning program with few stored reserves. In the work described above the oysters utilized for the work came into the hatchery environment with apparent high energy reserves and this may be a pre-condition for using an approach based on thermal shocks to stimulate rapid gametogenesis.

The question of supplemental feeding broodstock during the conditioning period has received much attention for most commercially cultivated species that have a hatchery based reared phase. Specific to *C. gigas*, there is little question that supplemental feeding results in increased fecundity in females and therefore more reproductive output per unit. Pacific oysters that were fed on rations approximating 4 and 12% of oyster body weight per day showed significantly higher reproductive output for oysters fed the higher daily ration (Delaporte et al., 2006). In this case, oysters fed the higher ration devoted 66% of their body mass to gamete production compared to 44% in oysters fed the lower ration. Observations for oysters fed a smaller percentage of body weight per day (often the case for oysters conditioned out of season in Pacific Northwest shellfish hatcheries) suggest that body tissues devoted to reproductive tissues are significantly less than that observed during the peak of natural reproduction. Comparing gonad development and use of stored reserves in oysters fed and starved over a 90-day period indicated that while gametogenesis occurred in the starved group, the rate of gametogenic development was significantly slower and the gonad 50% smaller than in the fed group (Liu et al, 2010). This

has been regularly observed in distinct genetic lines of Pacific oysters; comparative reproductive activity during artificial conditioning in the hatchery is s a fraction of that for the same line undergoing natural conditioning in the field (Joth Davis, unpublished observation). Similar findings had been well documented since the 1980's for Pacific oysters that have informed hatchery rearing efforts for this species for decades (Lannan et al. 1980; Muranaka and Lannan 1982; Chavez-Villalba et al., 2002). Finally, and related to the observations above, a number of researchers have identified significant genetic variation in the rate of gametogenesis in different genetic lines of Pacific oysters that result in significant variation in the rate of development and subsequent quality of gametes produced under a uniform conditioning program that relies strictly on a degree day approach (Lannan 1980; Hedgecock and Davis, 2007, J.P. Davis, unpublished observations).

Finally, the general practice for hatcheries to introduce oysters to the conditioning environment, typically in December and early January when light levels are near or at the annual low, may be amenable to improvement through the manipulation of light dark cycles. Oysters are typically slowly conditioned over a period of weeks to months before viable gametes are available, relying on a gradual temperature ramp and a supply of phytoplankton to stimulate the transition from storage physiology to reproduction.

It turns out that Pacific oysters may be significantly influenced by both increasing day length in concert with increasing temperature in the regulation of endogenous cues that influence the transition energy from storage to reproduction (Fabioux et al., 2005). While temperature has long been the focus for inducing out of season gamete development, the role of photoperiod has been largely ignored by the oyster industry and oysters are typically introduced to the conditioning environment with a focus only on temperature manipulations and their nutrition with no consideration of the influence of the light: dark cycle on triggering the transition from storage metabolism to gametogenesis. A better strategy might be to expose oysters that have come in from the field during winter months immediately to a 12:12 light dark cycle while increasing the ambient seawater temperature and increasing the level of feeding. Photoperiod should then be increased 30 minutes per day so that after 10 days the photoperiod experienced by oysters emulates that observed in late spring in the Pacific Northwest (17:7 light dark cycle). This strategy would require that oyster broodstock tanks be fit with lids and dedicated lighting that can be manipulated with timers. This approach warrants further attention by the oyster industry and may also be amenable to other species including geoducks.

The physiology of veliger larvae in Pacific oysters relative to nutrition and culture conditions that maximize production to the pediveliger stage.

Pacific oysters produce a trochophore larvae that initially use energy derived from the egg for early development before developing a velum and transitioning to a feeding veliger larva. The veliger stage or D-hinge stage is reached when the trochophore first secretes the prodissoconch I shell which occurs after approximately 18 hours of life in seawater at a temperature of 25°C. At the veliger stage they are then planktotrophic and gain much of their nutrition through ingestion of suspended algae. The veliger larvae initiates growth of the prodissoconch II shell, indicative

of a feeding larvae. Enlargement of the umbone from early to late umbone stage is also characteristic of this feeding stage. Larvae grow until they reach the pre-settlement stage or pediveliger stage at >275 microns in shell length, though size is somewhat variable at this late pre-settlement stage for unknown reasons. [A note on measuring oyster and other molluscan larvae; the maximal length (maximum anterior-posterior dimension) of the oyster larvae is the preferred metric though other measurements on larvae may also include a measure of lateral dimensions. Identification of the method used to measure oyster larvae is important to identify in any case.]

The pediveliger is characterized by a muscular foot that the larvae uses to probe for suitable settlement substrates and a pigmented eye spot. Time to this developmental stage is variable and largely dependent on nutrition but generally larvae reach the pediveliger stage between 13 and 18 days after fertilization at 25 °C. At this point, the larva settles, cements onto a suitable substrate and transforms into a juvenile via the process of metamorphosis. This is a critical juncture for oysters as they enter a developmental stage where the velum is shed, new feeding organs (ctenidia) develop and internal organs are shifted around to accommodate a benthic lifestyle. Feeding does not occur for a significant time frame while the ctenidia are developed. The non-feeding oyster also depends upon stored lipid reserves for energy during this critical juncture. The spat stage is then reached when new shell is deposited and the oyster initiates feeding on suspended materials though other components of metamorphosis continue for a number of days.

From the perspective of the hatchery operator, critical points during the oyster's early life history are survivorship bottlenecks. These include a) survivorship to the feeding early veliger stage (largely dependent on maternal investment in the egg), b) survivorship past approximately 100-micron shell length during the transition between early and late umbone stage and c) survivorship from pediveliger though settlement and metamorphosis to the juvenile stage.

A brief review of the physiology associated with larval production of Pacific oysters follows with a focus on the critical necessity for assuring nutritional requirements are met that maximize the benefit of maternal investment in the egg and nutritional requirements of the veliger larvae that maximize the potential for successful settlement and metamorphosis to the juvenile or seed stage in Pacific oysters.

Maternal investment in molluscan gametes is usually expressed in terms of mitochondrial investment in sperm and more importantly the amount of lipids contained in eggs or overall energy content in gametes overall (Bayne, 2019). For oysters the amount of lipid contained in the egg is highly correlated with survival of embryos to the veliger stage but egg lipid content does not appear to enhance larval survivorship once larvae initiate feeding (Gallager and Mann, 1985; Boulais et al., 2015). Once feeding is initiated in oyster larvae the quality and quantity of the algae used to rear oysters becomes a critical parameter to control. Of all the constituents contributing to a balanced diet for oyster larvae lipids are by far the most important. Lipid content in Pacific oysters is the best metric for determining both the health of larvae and is highly correlated with survivorship. Lipid classes that include (long chain) polyunsaturated fatty acids (PUFA's) are important to the health and growth *C. gigas* larvae. These include

arachidonic acid (C20:4), eicsapentaenic acid (C20:4) and docosahexaenoic acid (C22:6). All three of these highly unsaturated long chain fatty acids assist in energy metabolism and contribute to building phospholipids that are essential in increasing fluidity of cell membranes. Oysters in particular need DHA and EPA as an essential part of their diet for optimal larval growth. Because these lipid classes are considered essential, they need to synthesized by the oyster utilizing precursors obtained from the diet (da Costa et al., 2015 a and b). There have been extensive reviews on the biochemical characteristics of a wide variety of species microalgae, including information on changes in lipids induced by altering the culture conditions (Brown et al 1989, 1997; Tzovenis et al., 2003; Ponis et al., 2006a), including information for two algal species particularly nutritional for Pacific oysters (Ponis et al., 2006b). These are important papers that have the potential to materially improve the capacity for hatchery operators to increase production. Algal species typically utilized in hatchery operations in the PNW include *Isocrysis aff. galbana*, (clone T-iso; *Tahitian Isocrysis*), *Pavlova lutheri* (NMFS Milford Shellfish Laboratory clone 459), *Chaetoceros calcitrans, C. gracilis* and *Rhodomonas salina*. Microalgae for all these species are cultured following standard algal rearing methods and harvested during exponential growth phase. Optimal feeding strategies for culturing marine bivalves including Pacific oysters suggest a diet rich in lipids, including n-3 HUFAs (C22:6n3 and C20:5n3) as discussed above, but there is also the need to supply adequate carbohydrates for macronutrient supplies during development (Enright, 1984). Specifically, T-iso and *P. lutheri* have high levels of these two fatty acid constituents than *C. calcitrans* under normal phytoculture conditions (harvesting @ exponential growth phase) (Soudant et al., 1996; Brown et al., 1997).

As a recommendation, algal species grown for bivalve production need to provide better nutrition for Pacific oysters, including the provision of PUFA lipids necessary to enable the pediveliger oyster to survive the non-feeding period associated with metamorphosis to the juvenile stage. At this time, the vast majority of algal culture facilities associated with bivalve hatcheries culture microalgae under full light 24/7. This may not be an optimal approach for rearing microalgae if fatty acid production for DHA and EPA is desired. Preferably, a production schedule for microalgae should might consider either a 12:12 or 16:8 light: dark cycle for rearing diatoms for higher PUFA content (Fidalgo, 1998). Similarly, the amount of nutrient supplied *Isocrysis aff. galbana*, (clone T-iso; *Tahitian Isocrysis*) is important to monitor if increased PUFA's are desired. In this case, if urea is utilized as a source for nitrogen (as opposed to nitrate supplied typically in phytoplankton nutrient mixes) and cells are harvested at early stationary phase (as opposed to exponential phase as is typically the case for many hatcheries) then PUFA content may be enhanced. If culture conditions for microalgae are better managed for PUFA production, including the revision of light dark protocols this may assist in enhancing algal production having higher concentrations of the preferred long chain fatty acids.

The nutrition of early veliger larvae (beyond the straight hinge stage) in order to maximize the physiological condition of pediveliger larvae has been intensively investigated by many researchers. The use of formulated diets to improve the production of Pacific oyster larvae has also been a focus for many hatchery operations over the years. The possibility of utilizing

prepared feeds to replace or supplement live algae, during the winter months (typically the most difficult to rear live algae), has been a focus of research utilizing Saltonstall-Kennedy funding.

In an attempt to optimize seed production in the Pacific oyster, a number of focused studies on the use of Reed Mariculture Shellfish Diet (SD1800) were conducted as part of rearing studies designed to evaluate the possibility of using prepared diets in the production of Pacific oyster larvae. Reed SD1800 was selected for consideration because the manufacturer has focused on enhanced PUFA production. These studies were conducted over the period September 2017 through Summer 2019 at the hatchery production facility maintained by Baywater, Inc. and Pacific Hybreed, Inc. in Manchester, WA. The following experiment was typical of a number of experiments evaluating the efficacy of Reed Mariculture diets on efforts to produce Pacific oyster seed from egg to pediveliger stages.

Experiment 2 - RM SD1800 - Larval Rearing Studies: preliminary results and observations

A study comparing the survivorship and growth of Pacific oyster larvae reared on live algae alone, an equal combination of live algae and SD1800 and SD1800 alone was conducted. SD1800 was prepared using the protocol provided by Reed Mariculture. This consists of first suspending a volume of the liquid diet into a larger volume of fresh water and mixing well. This initial step is necessary prior to suspending the material into a head tank or other conveyance prior to feeding out. Also, because the SD1800 is preserved there is the need to suspend the material in the feeder tank. The use of air stones, while generally efficient in moving liquids, is not suitable for use with SD1800 because air bubbles tend to induce clumping. A more efficient approach is to suspend a small water pump into the head tank containing SD1800 and circulating the material continuously without introducing air bubbles into solution. This approach has proved effective in keeping formulated diets in suspension for many experiments. Baywater/Pacific Hybreed does not routinely use air stones to circulate feed solutions generally as introducing air into feed solutions (alive or preserved) has the tendency to introduce bacteria into the feeding system as well and is something to be avoided.

Methods

Ripe Pacific oysters were brought into the Manchester research facility in early Fall. Gametes were stripped using routine approaches from a total of 11 ripe oysters. Briefly, eggs and sperm were excised from the gonads of shucked adults by making incisions via scalpel in the gonad and rinsing gametes into clean beakers using a stream of filtered seawater. This approach yielded 9 females and 2 male oysters for use in the feeding experiment. Eggs from 8 of the 9 females were combined and counted by hand and suspended in a 5 L bucket to incubate for 60 minutes prior to fertilizing. Male gametes from two oysters were combined and suspended in seawater 10 minutes prior to fertilizing the egg suspension. Sperm motility was evaluated just prior to fertilizing eggs. Oyster eggs were fertilized with enough sperm suspension and evaluated microscopically to confirm that between 5-10 sperm were associated with each egg. Early development was observed long enough to ensure that fertilization of eggs had occurred.

Embryos were subsequently suspended in replicate 13 L volume buckets supplied seawater at a temperature of 23°C. Seawater supplied the bucket cultures was treated as follows: primary filtration (5 micron followed by 1 micron) followed by treatment through a cartridge containing and activated carbon bed. This preparation of seawater supplied larval cultures was maintained throughout the study. Five replicate buckets for the control group and the two experimental groups, respectively were stocked with embryos to assure an initial density of 30 embryos per mL seawater in bucket. Live algae was reared using standard approaches for rearing microalgae in a hatchery setting. Species of live algae included Tahitian *Isocrysis*, *Pavlova lutheri* and *Chaetoceros gracilis* mixed in an equal ratio of 1:1:1 by cell number. Cell counts were conducted by hand for live algae cultures. Feeding of cultures was achieved by premixing SD1800 per instructions. Dilutions of the SD1800 were made as described above. Numerical counts on the different species of live algae were made microscopically using a hemocytometer. Mixing of concentrate and the 1:1:1 ratio of live algae was subsequently made to achieve the control group (live algae only), the Experimental Group A containing an equal mix of live cells and concentrate (based on the assumption that there were 2x109 cells per mL) and the Experimental Group B consisting of concentrate only.

Throughout the study close attention was paid to providing each treatment group with an equal number of cells/ml algal diet across treatment groups. The following feeding protocol was used throughout the study according to the following protocol:

- D-hinge through Day 2 - 20,000 cells per mL, maintained twice daily
- Day 3 - Day 6: 30,000 cells per mL, maintained twice daily
- Day 7 - Day 10: 40,000 cells per mL, maintained twice daily
- Day 8 - Setting: 60,000 cells per mL, maintained twice daily

Results

Initial survivorship of embryos to the SH larval stage was consistent across replicates and at Day 3 all tanks were stocked at 75,000 larvae per tank. Throughout the study, consistently observed slower growth and lower survival in the group fed concentrate alone.

On day 8, larval counts showed that on average control cultures contained 31,786 larvae while Treatment A (50% live + 50% concentrate) cultures had a mean of 37,547 larvae and Treatment

B (concentrate only) contained an average of 10,240 larvae (Fig 32). This trend continued through day 17 when Control cultures contained a mean of 8,425 larvae, Treatment A contained a mean of 12,906 while Treatment B cultures contained a mean of 1,545 larvae per culture (Fig 33). Mean survivorship among the control and treatments from Day 3 to Day 8 varied from 11.23% for the control replicates to 17.21% and 2.06% for Experimental replicates, respectively.

Along with higher mortality observed in the concentrate only group, the larvae also grew more slowly. On Day 17 the majority of larvae in the live and live/concentrate groups were holding on a 100-micron screen while no larvae from the concentrate only replicates were holding on this mesh size. In fact, the majority of larvae were still passing through a 70-micron screen and

holding on a 48-micron screen. Larvae grown on concentrate only (Experimental B) examined microscopically appeared smaller, demonstrated lower motility and were lighter in color generally when compared to control and Experimental A groups.

On Day 19, Treatment B (SD1800 only) replicates were discarded due to the lack of larvae Remaining live and live/concentrate treatment groups were terminated on Sept 21 due to the loss of Treatment B replicates.

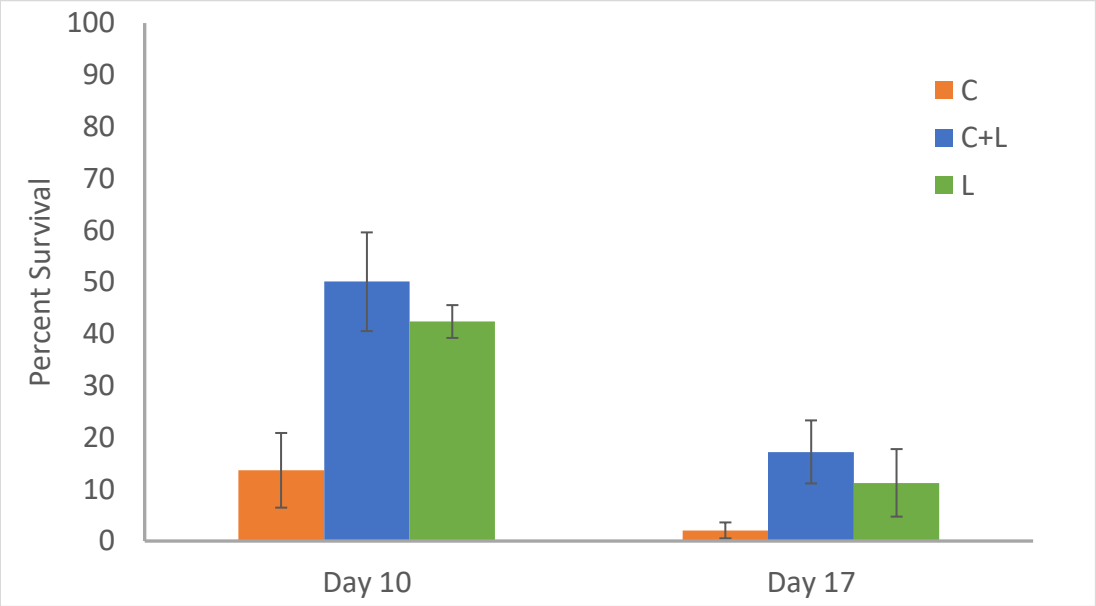


Fig 32. Survivorship of Pacific oyster larvae after 10 and 17 days fed different treatments. Results were statistically different for oysters reared on live diets as compared to SD1800.

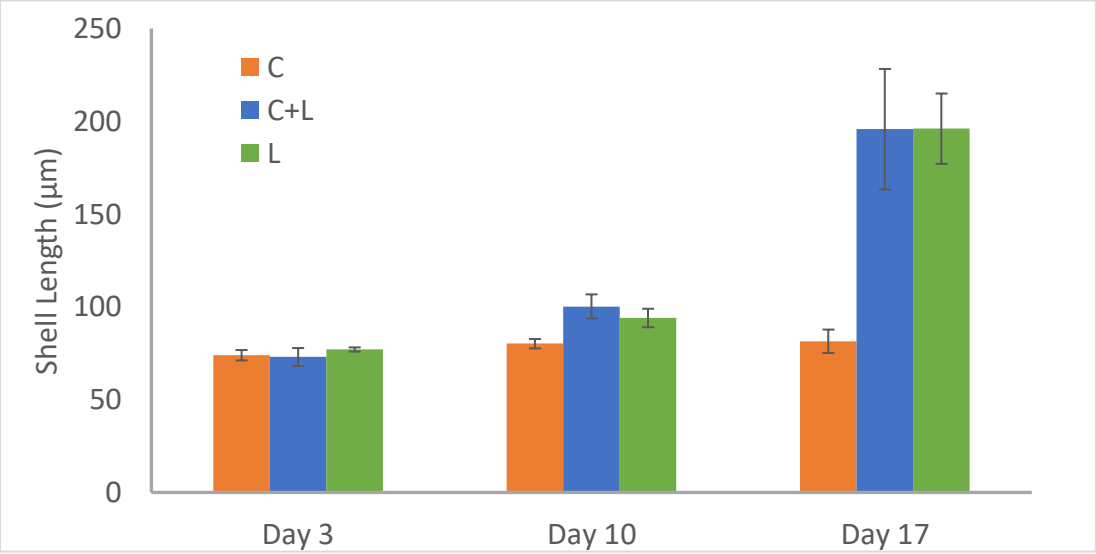


Fig 33. Growth of larvae (size at age) after 3, 10 and 17 days fed different diets. Diet treatments consisted of live algae only, 50% live algae + 50% SD1800 and SD1800 only. After 17 days, mean shell length of larvae fed a live and a SD1800 live combination were significnaly larger than for larvae fed SD1800 alone.

Discussion

Prospects for using the Reed Mariculture SD1800 for rearing larval Pacific oysters are not promising, based on these results. Additional work to replicate this study culminated in similar results over the course of the study period. The issue appears associated with either bacterial loading associated with the formulated diet or perhaps inhibitions associated with the preservative used to stabilize the feed. What is so interesting is that Eastern oysters (*C. virginica*) are routinely reared through metamorphosis using SD1800 alone in a hatchery in Alabama. Through the work we have accomplished at Baywater/Pacific Hybreed, discussions with this operator in conjunction with regular correspondence with Reed Mariculture focused on our inability to rear Pacific oyster larvae on the Reed Mariculture product. Other products produced by Reed Mariculture over the course of this project included Reed Mariculture Spat Diet (a test product not generally available) and more recently a new product referred to as LBB Frozen Shellfish Diet. We have observed similar results after extensive testing with these other products in rearing work with Pacific oyster larvae. The general result is that rearing Pacific oyster larvae on anything other than a combination of feeds that includes live algae have been disappointing. Pacific Hybreed is continuing testing, however on new products outside of funded work. The bottom line at this time is that rearing Pacific oyster larvae on SD1800 alone is not recommended unless live algae is not available. This result is based on multiple tests with SD1800 under a variety of circumstances at the Baywater/Pacific Hybreed hatchery facilities. The results indicate that larvae fed SD1800 only failed to thrive over the experimental period compared to larvae fed live algal cells only. The combination of live plus SD1800 as a 50:50 mix demonstrated better results than the treatment using SD1800 only with growth and survivorship intermediate to the Control and SD1800 treatments. Live algae still provides the best results in terms of both rate of growth and survivorship of larvae.

Physiology of pediveliger Larvae and adapting remote setting technology to optimize production of Pacific oyster seed.

Settlement and metamorphosis in Pacific oysters has received significant attention in the literature and has certainly been an intense focus for hatchery operators. Pacific oyster larvae are ready to settle and metamorphose at a shell length of >275 microns. As has been discussed, the nutritional status of the pediveliger going into this developmental phase with the supply of ample amounts of highly unsaturated lipids (along with carbohydrates and proteins) being of the utmost importance for the larvae to have in sufficient quantity prior to going into this critical developmental phase. The precise changes in the Pacific oyster larvae during settlement and metamorphosis have been described by Cannuel and Beninger (2006) in some detail and for oysters in the genus Crassostrea, generally (Baker and Mann, 1994). A description of the behaviors and physical description of Pacific oysters ready to settle and metamorphose is appropriate here.

Pacific oysters that have progressed through the larval phase develop a highly ciliated foot after about 14 days at a minimum and the larva is about 260-275 microns in shell length at this time. Other morphological changes in the pediveliger include the development of a round, pigmented area, referred to as the eye spot. The eye spot is helpful in identifying the transition from the late

umbone stage to the pediveliger stage, relative to the anticipated timing for settlement behaviors to begin. Morphological and physical cues that Pacific oyster larvae are competent to settle that are commonly observed microscopically include:

- The larvae has approached a shell length of 275 microns (holding on a 245-micron screen).
- The stomach of the larvae is brown in color indicative of being well fed.
- Small drops of oil (lipid) dispersed in the tissues of the larvae and may be discerned through the semi-transparent aragonite shell.
- The eye spot in the larvae/pediveliger assumes a diameter of between 10-14 microns.
- Larvae competent to settle tend to "swarm" and congregate in the water column. These may be characterized a long larval trails maintained by mucus threads secreted by the larvae.
- Larvae swim gregariously in agglutinated spiral columns just prior to settlement. Larvae typically swim to the top of the column near the water's surface and then cease swimming and fall back to the bottom before rejoining the spiral column.
- On the bottom and in contact with a substrate, the competent larva will crawl and/or rock from side to side on the substrate (often a glass microscope slide) with the foot extended.
- Careful observation will discern that the larvae just prior to settlement turns over onto its left side and attaches to the substrate (utilizing a byssal cement).

At this point, the larva has settled and soon initiates metamorphosis. This process has also been described (Cannuel and Beninger, 2006). Briefly, newly settled oyster (now referred to as a spat) jettisons the velum and foot and begins development of the ctenidia rudiment composed of 10-12 filaments. The eye spot degenerates quickly and the remaining tissues associated with the velum begin to disappear within 24 hours. Over a period of days, the gill filaments become increasingly ciliated and capable of capturing suspended food particles. These changes are discernable microscopically after 48-72 hours post settlement. Most importantly the newly settled spat initiates growth of new, calcite-based shell material. Prior to this point the shell material in the larva is composed of aragonite. The new shell in oyster spat increases the overall size of the oyster to > 420 microns.

Importantly, it has been well recognized that oysters within the genus, *Crassostrea* may metamorphose without first undergoing the settlement phase. The two behaviors are independent and may be exploited by the hatchery operator for the production of cultchless, or single oysters. When oyster pediveligers are exposed to the neurotransmitter, epinephrine development proceeds directly to metamorphosis, bypassing the settlement stage. Only small concentrations are necessary (10^{-5} molar) for brief periods (>10 minutes) are sufficient to induce this behavior (Coon and Bonar, 1987). Other chemicals may be of use in making cultchless oysters, including nitric oxide and L-DOPA (Coon et al., 1985). Further discussion on the use of neurotransmitters to create single oysters, including epinephrine and norepinephrine will be discussed in following sections of this report.

As discussed previously, the husbandry afforded to the care of the larvae (broodstock conditioning, larval feeding to maximize lipid storage and systems that accommodate the pediveliger transition from settlement to metamorphosis culminate in the production of spat that soon grow to the juvenile stage. The transition between the spat stage and the juvenile stage is arbitrary and characterized by significant mortality in spat.

Identifying other cues that may influence oyster larvae to settle and metamorphose with conspecifics has attracted researchers to consider the influence of natural noise. A recent study has documented increased settlement and metamorphosis of *C. virginica* in the presence of sounds typically encountered on oyster reefs. Recordings of reef noises and supplied to larvae via hydrophone under controlled conditions in the laboratory in Virginia resulted in higher numbers of larvae settling in the presence of reef noise compared to larval settlement in control groups not exposed to reef noises (Lillis et al., 2013). Provision of specific sounds and or specific acoustic wavelengths to oyster larvae competent to settle and metamorphose may provide additional cues that may serve the hatchery operator in maximizing larval settlement. This approach has not been applied to Pacific oyster larvae to date but provides an interesting approach that may assist in increasing the efficiency of setting eyed larvae.

An Evaluation of systems in use for setting Pacific Oyster Pediveligers for Spat and Juvenile Production

Setting systems for Pacific oysters vary according to their purpose. Two general systems exist; the first to accommodate spat on shell production and second to accommodate the production of single oysters for cultchless production. Both approaches rely on placing pediveligers into seawater systems over the period of days necessary to accommodate the developmental shift from the pediveliger stage to the spat and early juvenile stage.

Setting oysters for spat on shell production

Methods for spat on shell production utilize fiberglass tanks maintained either indoors or outdoors that are supplied with filtered seawater (20 micron) and plentiful aeration. On the US west coast, seawater tanks used for setting may be of any volume but systems in production are in the range of 3000 to 30,000 L. Seawater is pumped and heated to 20 - 25°C and supplied to tanks that are typically maintained for a minimum of 48 hours before draining and refilling. Tanks supplied with seawater that is added and drained continuously are also in common use. In this case a banjo style screen accommodates the discharge of seawater form the tank and precludes the loss of larvae. Flow through tanks also preclude the necessity for draining and refilling tanks at regular intervals and alserve to maintain the temperature of the seawater at the prescribed temperature. Static tanks must be supplied with an electric immersion heater to maintain the temperature of seawater in the tank. Tanks are then supplied with settlement substrates. In much of the United States, settlement substrates consist of clean oyster shells contained in plastic mesh bags. There are typically about 300-350 oyster shells in each cultch bag. Bags are stacked into the settlement tank and allowed to condition for a day or two prior to introducing pediveligers. Pediveligers are added to the settlement tank in sufficient numbers to

enable the cultch to attract, on average between 10-30 spat per shell. The number of larvae added to the tank is dependent on the likelihood of a set and hatchery preference.

A rule of thumb for stocking tanks may be as follows. Assuming a setting tank accommodates 100 shell bags with each bag containing 300 pieces of shell. The total shell substrate available is therefore 30,000 shells. If the desired set is 10 spat per shell then 300,000 pediveligers must successfully be accommodated on the available substrate. Accommodating settlement on the sides and bottom of the tank is highly variable and must be accounted for. Assuming the rate of settlement is 30% of the total number of pediveligers added. In this case, to accommodate mortality and tank loss, 2 million pediveligers would be added to the settlement initially. This number is highly variable and depends on the operator's experience. Larval survivorship to the spat stage may be low (<5%) but often exceeds 30% and is therefore highly dependent on the relative quality of larvae introduced to the system initially.

Receipt of pediveligers and best management practices for optimizing settlement success
Pediveligers may either be produced by the hatchery and utilized internally or purchased from other hatcheries. Pediveliger larvae received via overnight shipment arrive cool (5-10 C) and must either be set immediately or stored in a "warm refrigerator maintained at 6-8°C. until use. Storage of larvae in the refrigerator is addressed below. Larvae typically arrive in an insulated box with the larval ball wrapped in bed sheeting that has been dampened with seawater. A loosely contained in a plastic baggie or paper toweling. in in any case, upon receipt the pediveligers must first be evaluated for their relative state of competence. First, the pediveligers are allowed to come to room temperature if they have been refrigerated and left for a period of 45-60 minutes. Second, pediveligers are typically dispersed into an adequate volume (1,000,000 larvae per 10 L volume) of seawater maintained at 25°C. Once pediveligers become active, ideally swimming up into the water column in spiral columns while producing prodigious amounts of mucus, an evaluation of competence to settle should be made. This is easily accomplished by suspending several pieces of clean oyster shell into the bucket and examining the shells every five minutes. If oysters are observed to be cementing onto the test shells in reasonable numbers the larval group is fully competent to settle and metamorphose to the juvenile stage. For spat on shell operations this is accomplished by simply adding larvae to the preconditioned setting tank. Pediveligers will swim and/or settle onto available substrates over the ensuing 24 hours. Vigorous aeration supplied to the tank aids in dispersing the larvae amongst the shell-filled cultch bags. Tanks are typically covered to maintain the system in dark to semi-shaded conditions. Accommodation for the number of larvae that settle onto the sides and bottom of the setting tank compared to the numbers setting on the oyster shell substrates must be made as settlers on tank sides and bottom will be lost from the cohort.

After 24-48 hours live algae is added to the tank to supply new settlers with nutrition though many operators supply algal feed to the tank at the onset of the settlement period. In this case, algae is therefore also available to larvae that were not yet competent to settle and metamorphose. The addition of microalgae, however, adds organic substrates to the tanks and

uneaten algae may generate unwanted bacterial growth that may contribute to declining water quality.

Experience by the hatchery operator and the level of competence exhibited by the larvae will dictate the return of seed to the operator. After 1-2 days the operator will hopefully observe a viable set on the oyster shells contained in bags. A typical set will consist of 8-20+ spat per shell.

Once the spat grow to a size of 2-4 mm on the cutch shells the operator often moves the shell bags to an intertidal beach to grow and harden. Hardening in this case refers to the tendency for the oysters to thicken their valves once exposed to regular aerial exposure during low tides. Later, the spat (now considered juveniles) are counted on a number of test shells to estimate the return for this cohort of seed. Moving spat to the beach also frees up the setting space for additional cohorts of larvae.

Setting Larvae for cultchless or single oyster production

There are a variety of systems in use for setting oysters for single oyster production. Typical systems are characterized by trays suspended in troughs, flexible plastic sheets, down well systems, bottles or cylinders all supplied with running seawater. The description and use of these different systems will be considered below. A decision faces the operator, however of whether to set oysters with the use of a pre-treatment consisting of a brief exposure to an epinephrine bath to induce oysters to initiate metamorphosis without the necessity to settle and cement onto a substrate.

For this discussion, setting systems for oysters will first be considered without the use of a neurotransmitter. All of the systems will require a supply of micro-cultch to supply the system a settlement substrate to accommodate pediveliger's requirement to settle and cement to a hard substrate. Micro-cultch is made by pulverizing clean and dry oyster shell and passing the shell fragments through a series of screens to capture fragments of a specific size class. Ideally, shell fragments between 300-400 microns are retained for use in setting. The shell fragments need to be large enough to accommodate a pediveliger larva probing for a suitable substrate to settle upon but small enough to preclude more than one larva occupying the fragment. Otherwise, two or more oysters may occupy the same fragment and constitute a cluster, thus defeating the objective of producing single oysters. Once micro-cultch is available the setting system can consist of any number of designs

a) Setting in trays The most straight forward approach to setting Pacific oysters includes the use of trays. These are constructed of a wooden or fiberglass frame and covered with nytex mesh with a porosity of 180 micron. Trays are suspended in larger tanks or troughs that are supplied with running seawater at the appropriate temperature (20-25 °C). Micro cultch is spread on the bottom of each tray, ideally distributed as a thin layer. Larvae competent to settle and metamorphose are added to the trays and left for 2-4 days. Trays are typically covered to maintain the larvae in shaded or darkened conditions. Supplemental feeding may be added as described above for cultch based systems. If pediveligers are competent to settle and metamorphose, following the cues described above, spat will be observed attached to shell

fragments after 24 hours. The spat are allowed to grow until they hold on a 500-micron screen (4-6 days). At that point, the spat should be rinsed using a gentle spray of filtered seawater through a 450- or 500-micron screen to remove micro-cultch that was not colonized and any larvae that did not settle. This is also the opportunity to remove debris from larvae that died over the settlement period. Feeding of microalgae should be initiated after 24 hours with cell counts maintained in the range of 100,000 – 150,000 cells per ml of volume to enable spat to initiate shell new dissaconch shell growth. The diet should consist of a mix that includes *Isocrysis aff. galbana*, (clone T-iso; *Tahitian Isocrysis*), *Pavlova lutheri* (NMFS Milford Shellfish Laboratory clone 459), *Chaetoceros calcitrans*, *C. gracilis* and *Rhodomonas salina*, among other candidate species.

Stocking density for screen-based systems are based on the area devoted to micro-cultch. A simple rule of thumb is to stock larvae at no more than 100 pediveligers per square cm of screen area. A screen having an area of 5000 square cm could therefore be stocked with 50,000 pediveligers. Higher or lower stocking densities may also be used but over-crowding is to be avoided to enable maximal setting success.

b) Setting in downwellers In the Pacific Northwest many hatchery operations set oysters directly on screens that are integral to a downwelling unit. Typical systems are characterized by silos of varying diameter between 4" and 30" that are suspended in a trough or box supplied with filtered seawater. Typically, the box system has an option for operating either in upwell mode (seawater passes up through the bottom of the screen and out of the silo) or in down well mode where the water passes through the silo exiting the bottom of the screen. Downwell mode is usually used for the initial setting phase for Pacific oysters. Screens are outfitted with mesh having a porosity of 180 microns. As described above for screen-based systems, the stocking density of pediveligers for down well based systems should be maintained at about 100 pediveligers per square cm. Systems commonly in use in the Pacific Northwest utilize three screen upwell/down well boxes. Each screen has a screen area of about 2000 square cm and should be stocked with no more than 200,000 pediveligers per screen, or 600,000 pediveligers per box. A typical return to the spat stage using a down well based system should be between 10-40% survivorship from the pediveliger stage. Thus, a down well/upwell box system should typically return between 60,000 to 240,000 seed under satisfactory conditions. Typical success rates are rarely in excess of 40% and are often below 10%, however.

Once setting and metamorphosis is complete, the process for handling new oyster spat is similar to that described above for tray setting. Oysters are rinsed with saltwater and screened to remove unused micro-cultch and debris and fed cultured algae until the juveniles reach a size suitable for out planting into nursery systems (typically a size that holds on a 2000-micron screen). Rinsing of screens should occur daily, initially using seawater as source for spray downs and cleaning. The use of freshwater is to be avoided until the seed are week old. At that time, a daily freshwater rinse is preferred if there is any evidence of post set mortality. Feeding rates should adhere to similar levels recommended above for screen-based systems. The use of silo-based systems is that the feeding rate of spat may be estimated by estimating the depletion rate for algal cells as they pass through the system. Though settlement of algal cells is unavoidable, a feed rate

set so that the reduction in particle concentration from the incoming flow compared to the seawater exiting the system is at least 20% of incoming particle density is preferred.

Once the spat attain a size of 1000-1200 microns, the direction of flow on down well/upwell boxes may be switched to the upwell mode. This will significantly reduce the amount of fouling on the screen surface and enable faster growth of the seed once they attain this size.

c) Setting on flexible plastic sheets While use of flex. plastics is not regularly used in the Pacific Northwest, their use is widespread in Europe and Asia where Pacific oyster culture is widely practiced. In this case, flexible plastic or mylar sheets are suspended vertically in setting tanks and pediveligers added to the system as described above. In this system, oysters are allowed to settle and metamorphose onto the plastic sheets and to grow for 1-3 weeks, depending on productivity and growth rate. At that time, the sheets are flexed to enable the oyster spat to pop away from the settlement substrate as single oysters. Oysters are subsequently reared as single oysters in trays or upwellers as described above.

d) Use of epinephrine to produce single oysters Single oyster production using the hormone/neurotransmitter epinephrine may be used to produce single Pacific oysters without the necessity to provide micro-cutch during the settlement/cementation stage. As described earlier, a small amount of epinephrine supplied to pediveligers, otherwise competent to settle, will induce the larva to bypass the cementation phase and directly initiate metamorphosis. This has the advantage of producing single oysters without the need to supply micro-cultch to the setting system. A concentration of epinephrine of between 10^{-4} – 10^{-5} molar in seawater provides a satisfactory working concentration (Coon et al., 1985; Coon and Bonar, 1987). Pediveligers competent to settle and metamorphose are introduced to the mix and incubated with regular plunging over a 20-130-minute period. No more than 100,000 pediveligers should be treated in a one-liter volume of epinephrine/seawater mix. Best results are obtained if the epinephrine treatment occurs in a darkened room. Oysters are rinsed in filtered seawater following the treatment and resuspended in seawater. Any larvae that swim up following the treatment may be returned to the larval rearing tank as these are larvae that were not competent to settle. At the time of the treatment. Larvae may be re-treated a 1-2 days later with a freshly made epinephrine seawater mix and the seed reared normally. The epinephrine seawater mix must be disposed responsibly. Oysters remaining on the bottom of the vessel (no longer swimming) may now be reared as spat utilizing a screen-based nursery system as described above.

Conclusions

The aquaculture sector focused on the production of Pacific oysters has experienced a number of years of reduced production of juveniles that have significantly impacted shellfish farm operations. There are opportunities to utilize published information that has either been neglected or ignored by the hatchery sector as it pertains to the production of oysters. This may be in part due to the relative ease of rearing Pacific oysters in year's past. Today, however for reasons not completely understood, the production of oyster seed consistently has been difficult for many hatchery operations.

There needs to be a renewed focus on the factors that assist in the successful rearing of Pacific oysters. These include better attention to the conditioning process for the routine production of gametes that can result in higher quality eggs being made available for larval and seed production. Attention to specific biochemical parameters related to the investment in eggs of highly unsaturated fatty acids appear to be very important. These include:

- Approaches to algal rearing techniques with special attention to light cycles and nutrients that assist in boosting algae stores with PUFA's
- Selection of broodstock from the field that have undergone a natural cycle of nutrient investment and avoidance of bringing oysters into the hatchery that are below weight and condition.
- Attention to broodstock maturation approaches that focus on harvesting gametes while oocytes are in growth phase and avoiding gametes that are overripe and undergoing resorption.
- Preliminary information suggests that Pacific oysters can be rapidly induced to produce viable gametes with after just two weeks following exposure to a 28°C temperature shock for four days with further conditioning at 22 °C. An approach based on a rapid maturation protocol, as described here may assist in producing gametes for better larval and seed production.
- Broodstock management for gamete that ensures harvest of eggs while still in active growth phase is to be preferred over the use of gametes that may be undergoing resorption.

Remote setting technology for Pacific oysters was reviewed with a focus on the techniques used by the Pacific Northwest shellfish industry today for both spat on shell and approaches for single oysters. A focus on attention to the physiology of pediveligers to ensure that metrics are used to better assess competency are critically required, along with instituting better controls over feeding and setting density for screen based systems. A short review of best management practices for handling a new set of oysters is provided, based on current industry practices for different setting techniques.

- For initial production of single oysters, setting densities for larvae above 100 pediveligers per square cm should be avoided.
- Results of experiments using a commercially available formulated diet (Reed Mariculture, Inc.) were not positive due to unknown reasons as the products have been tested for other species of oysters with positive results. The nutritional profile of the algal species used in the Reed Mariculture products should result in better nutrition provided to broodstock and larvae. For the case of rearing larvae on Reed SD1800, this proved not the case.

Products and Dissemination of Project Results

Industry mangers are the primary target of the information generated by this project, as they have the highest investment in seed and adult Pacific oysters. The information generated via the project includes the following major products, as well as the "Remote Setting: lessons learned and future needs" workshop that was facilitated during the project period:

A Project Summary (see Appendix A) outlining the problem and proposed work during the project period was available throughout the project period on the PSI website and available in hard copy at the Pacific Coast Shellfish Growers Association (PCSGA) annual conferences from 2016-2019 and Washington Sea Grant (WSG) Conference for Shellfish Growers 2016-2019.

A White Paper summarizing the major workshop findings and survey findings, the results from Aquatechnics diagnostic exams, and Pacific Hybreed literature review and seed culture method research was developed by PSI staff to effectively distill the major findings from this project into an easily consumable format for the community of shellfish growers and seed producers. The drafted form is currently pending review by project partners, and will be available through the PSI official website. Hard copies of the white paper will be made available to shellfish growers at the WSG Shellfish Growers annual conference in the Spring of 2020.

A Mortality reporting form (see Appendix B) was developed for growers to record seed and adult mortalities and submit to PSI staff. The mortality reporting form came in response to several, disparate reports of adult mortalities on Washington farms. From 2018-2019, six total farms pursued diagnostic testing through Aquatechnics, despite several verbal accounts of mortalities to PSI staff and to staff at Washington Sea Grant. As such, there was a demonstrated need for an official reporting form to categorize the occurrence of mortality events in hatcheries and farms. The form was presented by PSI during the PCSGA annual conference in Portland, Oregon 2019 to an audience of seed producers, regulators, and researchers. Additionally, the form was sent out to all growers that took advantage of diagnostic services during the project period. It was made available on the PSI website in August 2019, and will continue to be available indefinitely. The form contains the contact information of Aquatechnics testing services for growers interested in further oyster health diagnostics.

Three Presentations developed by project partners were given to audiences of researchers, industry managers, and regulators during the project period. Two presentations were given by Dr. Ralph Elston (Aquatechnics) at WSG Annual Conference for Shellfish Growers in 2018 and at the PCSGA 2019 Annual Conference. Dr. Elston's presentations included diagnostic exam results and findings from the project. PSI staff presented the final results of the project at the 2019 Pacific Coast Shellfish Growers Association.

This Final Report will be available on the PSI website at www.pacshell.org. Additionally, it will be shared with all project partners and industry collaborators. The products of this project should provide producers with information relevant to decision-making and provide the industry as a whole with a tool for categorizing mortality data for future analysis.

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