

## **Emergency Plan to Save Oyster Production on the West Coast January, 2009**

A Collaborative Proposal Prepared by the Pacific Coast Shellfish Growers Association, Whiskey Creek Hatchery, Taylor Hatchery, Pacific Shellfish Institute, Willapa-Grays Harbor Oyster Growers Association, Lummi Indian Tribe Hatchery, U.S. Department of Commerce (NOAA Aquaculture Program), Northwest Fisheries Science Center (NOAA), U.S. Department of Agriculture (ARS and CSREES), Oregon State University, AquaTechnics, Inc., and the Nature Conservancy

### **The Problem:**

For the past three years, water quality conditions in the Pacific Ocean off the Oregon and Washington coasts; and adjacent highly productive estuaries including Puget Sound, Willapa Bay, and Netarts Bay, have severely impacted hatchery production of seed oysters upon which both large and small farms depend. Simultaneously, wild sets of oyster seed that make up the back-bone of the oyster industry in Willapa Bay, the single largest oyster producing region on the West Coast, have been virtually non-existent for the past four years.

These conditions have led to dire economic consequences for two of the four hatchery operators that produce oyster seed for farmers, including the largest producer of oyster larvae on the West Coast, Whiskey Creek Hatchery, which accounts for approximately 75% of all larvae utilized by farmers. The environmental conditions contributing to the lack of wild seed set presents an even more challenging problem.

Another year without adequate seed will likely result in the collapse of several businesses. The need for immediate action is critical.

### **Solutions:**

This Emergency Proposal includes funding requests for identified short-term solutions that can be implemented within the next few months. The most critical task at hand is to retrofit hatcheries. Small-scale experiments have already been conducted that indicate effective water quality treatments can be implemented within closed hatchery systems to optimize oyster seed survival. Another critical component that has been identified both for the short and long terms is to establish monitoring programs in key estuaries to better understand the environmental forces at work. Identification of disease resistant oyster strains and better identification tools to detect disease causing bacteria are also included in this Proposal. (See Appendices 1-4)

### **Economic Consequences:**

As of 2005, the farm-gate value of oysters, clams, geoduck and mussels on the West Coast of the U.S. was \$111 million, with oysters accounting for 76% of all shellfish produced. West Coast farms provide over 3,000 family-wage jobs in rural coastal communities. Including related service sectors and suppliers, the total economic contribution of the shellfish farming community on the West Coast is estimated at \$278 million annually. Based on the past four years, and assuming nothing is done to intercede, the total contribution of shellfish aquaculture to the economy is projected to be reduced by 30 percent in the year ahead – approximately \$83 million - with even steeper declines in the years ahead as broodstock populations in the wild become more depleted.

### **Environmental Consequences:**

Reduced populations of bivalves, a critical keystone species in the marine ecosystem, will have significant

consequences. The reduction in water filtration by these shellfish will impact water quality, and diminished shellfish assemblages will reduce forage and refuge opportunities for the host of marine flora and fauna that utilize shellfish beds. Like the proverbial “canary in a coal mine” the seed shortage crisis has consequences that extend up the food chain, and our ability to more fully understand the dynamics at work will provide benefits that go well beyond shellfish farmers and their local communities.

**Identified water quality/hatchery problems:**

Shellfish hatcheries have historically used coarsely filtered but otherwise untreated seawater for larval culture with few problems, and larval shellfish have thrived in water in the Pacific Ocean and coastal estuaries. Upwelling of deep, cold, nutrient-rich water from the continental shelf off the coast of Oregon and Washington is typical during summer months in this region and drives high primary productivity. Since 2003, however, higher than normal upwelling increased the extent and intensity of intrusions of deep acidic, hypoxic water off the Oregon and Washington coasts, and contributed to the formation of persistent dead zones. These events have resulted in fundamental changes in the character of our coastal bays, which contribute to high larval mortality throughout the entire year.

These fundamental changes in seawater quality influence a host of complex chemical interactions, many of which are not fully understood. However, recent research has identified at least four potential stressors that adversely affect shellfish larvae:

- Larval and juvenile shellfish are highly sensitive to acidic (low pH) seawater because their shells are formed from calcium carbonate, and dissolves when pH is low.
- Because this hypoxic and relatively acidic up-welled water is coming from deep basins and is cold (8 - 10 oC), it is saturated with dissolved gases such as carbon dioxide and nitrogen while at the same time being low in oxygen as a result of biological decomposition in the benthic zone. When hatcheries heat this gas-saturated seawater to 25 - 28 oC in order to meet the temperature requirements of young shellfish, the seawater becomes super-saturated. Preliminary experiments indicate that oyster larvae are very sensitive to gas super-saturation under these conditions.
- A third problem for shellfish hatcheries is the recent increase in the prevalence of a pathogenic bacterium (*Vibrio tubiashii* or *Vt*) that seems to out-compete other, more benign species in this distorted environment. *Vt* infections are lethal to shellfish larvae and juveniles. High levels of mortality in shellfish hatcheries and in the wild have been associated with high levels of *Vt* in 2006, 2007, and intermittently in previous years, such as in 1998 when environmental conditions favored disease outbreaks.
- There is potential for further stress to oyster seed given the difference between water conditions in the hatcheries where larvae are produced, and quality of water found in the remote settings where larvae set onto cultch (“mother shell”) are planted in the natural environment for grow-out.

These adverse environmental conditions - low pH, gas super-saturation, high *Vt* infections, and the associated complex effects on seawater chemistry - constitute a “perfect storm” for Pacific Northwest shellfish hatcheries and growers that depend on natural set oyster seed, bringing the industry to the brink of collapse. It is not understood how these, and likely other, stressors interact, but it is clear that these factors are somehow combining to decimate shellfish larvae and juveniles. To further illustrate the seriousness of the situation, oceanographers such as Dr. Richard Feely, world-renowned NOAA expert on ocean acidification and global warming, predicts that oceanic conditions will not improve in the near term, potentially rendering shellfish hatcheries inoperable. This, combined with lack of wild seed set, will lead to the collapse of the oyster industry unless mitigation measures are developed and implemented immediately.

Preliminary research at Oregon State University, in conjunction with hatcheries, Aquatechnics Inc., and

other institutions, indicate that comprehensive treatment of seawater incoming to hatcheries can potentially mitigate these environmental perturbations. Seawater treatments include degassing the heated water, micro-filtration, pH adjustment, and ultraviolet or ozone sterilization to kill *Vt*. While these technologies have been implemented on an experimental scale for research purposes, commercial shellfish hatcheries have been unable to fully implement and test these methodologies on a commercial production scale. In addition, recent experience indicates that some genetic strains of shellfish are more resistant to these stresses than the current breeding stocks. Unfortunately, due to the downward spiral of production and the associated declines in revenues at the affected hatcheries, private funds are inadequate to intensify monitoring of incoming water at hatcheries in a systematic and consistent manner at a time when these critical data are especially important for identifying the cause(s) of this catastrophe, which might also include other factors yet to be identified.

This proposal is broken down into four components, in order of identified priorities:

Priority 1: Equip hatcheries for water treatment & evaluate efficacy.

Principal Investigators: B. Eudeline, Taylor Shellfish Hatchery; Alan Barton, Whiskey Creek Shellfish Hatchery; Chris Langdon, Oregon State University

See Appendix 1 for details.

Funding requested \$408,764

Matching funds \$276,108

Priority 2: Monitor and record incoming seawater quality and bacteria concentrations, both at hatcheries and at other commercially important bays, such as Willapa Bay, where wild seed production is critical.

Principal Investigators: B. Eudeline, Taylor Shellfish Hatchery; A. Barton, Whiskey Creek Shellfish Hatchery; J. Edwards, Coast Seafoods; L. Oberlander, Lummi Indian Nation, Ted Kuiper, Chris Langdon, OSU; Bill Robertson.

See Appendix 2 for details.

Funding requested: \$340,500

Matching funds: \$117,000

Priority 3: Short-term genetic studies to identify oyster broodstock resistant to adverse environmental conditions.

Principal Investigators: Mark Camara, USDA/ARS; Chris Langdon, OSU

See Appendix 3 for details.

Funding Requested: \$131,228

Matching funds: \$61,500

Priority 4: Develop on-site (in the field) specific detection methods for *Vibrio tubiashii* and its toxins.

Principal Investigator: Claudia Hase, OSU.

See Appendix 4 for details.

Funding Requested: \$79,633

TOTAL FUNDING REQUEST: \$960,125

TOTAL MATCH: \$534,241

TOTAL PROJECT COST: \$1,494,366

#### Timelines

December/January Meetings with key agencies, legislators and stakeholder groups.

February, 2009 Critical date for commitment of funding to implement plan.

February-April Implementation of retro-fits, planning of monitoring.

April 2, 2008 Proposal operational

For more information contact:

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## **Appendices for “Emergency Plan to Save Pacific Coast Molluscan Shellfish”**

Appendix 1: Installation and evaluation of commercial scale water treatment systems to improve larval production at Pacific Coast shellfish hatcheries. Pages 2- 10

Appendix 2: Coast-wide monitoring of commercially important bays and estuaries in the Pacific Northwest. Pages 11- 16

Appendix 3: Rapid screening of oyster germplasm resources to identify families resistant to adverse environmental conditions. Pages 17-22

Appendix 4: Development of a rapid “dipstick” assay for on-site detection of *Vibrio tubiashii* in commercial shellfish hatcheries. Pages 23-29

## **Appendix 1**

### **INSTALLATION AND EVALUATION OF COMMERCIAL SCALE WATER TREATMENT SYSTEMS TO IMPROVE LARVAL PRODUCTION AT WEST COAST SHELLFISH HATCHERIES**

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AMOUNT REQUESTED: \$408,764  
MATCHING FUNDS: \$276,108

#### **BACKGROUND AND RATIONALE**

Commercial production at Whiskey Creek Shellfish Hatchery in Netarts, Oregon, and at the Taylor Shellfish Hatchery in Quilcene, Washington, has been in rapid decline over the last three years. Whiskey Creek Shellfish Hatchery traditionally supplies approximately 80% of oyster larvae sold to independent growers in the Pacific Northwest, and poor production in the past two years has precipitated a critical shortage of oyster seed for many farms in Oregon, Washington, and California. Taylor Shellfish is a vertically-integrated company, and the majority of larvae produced in their hatchery is used in-house to supply seed for their own company, the largest producer of farmed shellfish in the U.S., and one of the world's largest shellfish farming companies. The production problems at both Whiskey Creek and Taylor hatcheries have translated into significant job losses at oyster farms on the West Coast, and unless normal larval production is immediately restored, the oyster industry, and the \$278 million it contributes to the economy annually, will continue its crippling decline.

Therefore, immediate action must be taken to improve seawater quality in these important commercial

hatcheries. Enhanced seawater treatment systems must immediately be implemented on a commercial scale, based on research and development conducted during the 2007-2008 growing seasons. In early 2008, preliminary efforts were made to improve water quality at Whiskey Creek, through the installation of a commercial-scale water treatment system. This system was modeled after a treatment system developed in 2005 by the Molluscan Broodstock Program (MBP), a research hatchery in located at Oregon State University's Hatfield Marine Science Center, under the direction of Dr. Chris Langdon. The design of this system was targeted at mitigating the effects of *Vibrio tubiashii*, a bacteria which has been linked to severe mortality events in commercial hatchery settings. Although *Vibrio tubiashii*(*Vt*) is a naturally-occurring bacteria, its prevalence in coastal bays has increased dramatically from 2006-2008, and numerous observations have shown *Vt* to be the dominant species of bacteria present in Netarts Bay.

Production in the 2008 season was extremely poor, and represents the worst production season in the thirty year history of Whiskey Creek Shellfish Hatchery. A limited level of consistent production was achieved in June 2008 using the seawater treatment system, but the entire hatchery experienced a catastrophic mortality event in mid-July, which was coincident with a period of strong coastal upwelling. This event forced personnel at Whiskey Creek to reassess the problems the hatchery, and to understand that *Vt* was not the sole source of stress facing larvae in the hatchery.

This assertion is supported by hatchery personnel at Taylor Shellfish Hatchery in Quilcene, Washington, which also experienced extremely poor oyster larvae production in the 2008 season. The Quilcene hatchery is located on Dabob Bay, an interior bay of Hood Canal. As a result, the characteristics of seawater are quite different from those observed at Whiskey Creek. *Vibrio tubiashii* concentrations in Dabob Bay have not reached the levels observed in Netarts Bay, and although *Vt* is certainly a stressor for larvae at the Taylor Hatchery, its presence has not been shown to be directly correlated with severe mortality events.

These observations from both hatcheries have redirected research efforts in the second half of 2008, which are now focused on understanding the fundamental shifts in seawater chemistry that are severely impacting our coastal bays. The intense upwelling event observed at Whiskey Creek in mid-July brought extremely acidic seawater onto the continental shelf, and pH measurements of 7.5-7.6, (8.2 is healthy), were recorded during the mortality events observed in the hatchery. Taylor Shellfish has also experienced extended periods with pH as low as 7.3-7.5, due at least in part to the unique characteristics of Hood Canal. Efforts to correct pH at Whiskey Creek have produced a limited measure of success, and dramatic improvements in survival of mussel and manila clam larvae were observed in July-August of this year. However, rapid correction of pH did not eliminate stress on oyster larvae, and production remained quite low during a two-month period of steady pH in the hatchery. This species-specific response suggests a much more subtle change in seawater chemistry has occurred in these commercially-important coastal bays.

Discreet measurements of low oxidation-reduction potential (ORP) in Netarts Bay suggest that high concentrations of reducing compounds are periodically present, some of which (e.g. sulfides and sulfites) might be toxic to oyster larvae. This condition could also explain the increase in prevalence of *Vt*, which thrives in reduced environments. Ongoing research also suggests that upwelling coastal waters contain increased concentrations of dissolved gases that may be harmful to oyster larvae.

Although it is currently unclear which of these, or other stressors, is most affecting commercial hatchery production, a number of commercial technologies exist that address these seawater quality issues in a wholesale manner. Similar issues have been faced for years in recirculating aquaculture of marine fish species, and solutions have been developed and implemented in large commercial production facilities.

Therefore, we propose to augment existing seawater treatment systems at Whiskey Creek and Taylor Shellfish with these technologies, and begin the 2009 growing season with a comprehensive set of water treatment options. Although the ultimate goal of our efforts will be to define and correct specific water quality problems, the urgency of our seed supply crisis dictates that we adopt a wholesale approach to seawater treatment to best ensure production in Spring of 2009. Whiskey Creek, a privately-owned hatchery, has limited operating capital remaining, and cannot likely survive without production in the early months of 2009. Another early season without seed production from these hatcheries will spell disaster for the oyster industry, so it is imperative that we put our best foot forward to start the 2009 growing season.

With the following suite of treatment options in place, we propose a systematic approach to understanding specific water quality issues in each hatchery. We will conduct continuous monitoring of seawater quality, including a suite of chemical parameters, along with routine monitoring of pH, ORP, temperature, salinity, and dissolved oxygen. Monitoring stations will be installed both before and after each stage of seawater treatment, to understand how each process produces specific changes in water quality. Simultaneous bioassays with oyster larvae will be conducted, to understand how these improvements in seawater quality ultimately contribute to improved survival and growth of larvae in the commercial hatchery setting.

#### PROJECT APPROACH

Purchase and install seawater treatment systems

Research conducted in 2008 has defined a list of treatment options that have proven effective in small scale larval bioassays. Larger versions of these systems must be purchased and installed immediately, for use in commercial production in early 2009.

Purchase and install water quality monitoring/sampling systems

Instruments to continuously monitor pH, ORP, temperature, salinity, and dissolved oxygen will be installed in the hatcheries both before and after water treatment, and data loggers will be deployed remotely in the bays outside each site. Seawater chemistry will also be monitored daily in the hatcheries to understand trends in water quality that may affect survival and growth of oyster larvae, and to understand changes induced by seawater treatment systems. In addition, frequent samples will be sent to the College of Ocean and Atmospheric Sciences at Oregon State University for more detailed analysis of seawater chemistry. Weekly samples will also be shipped from hatcheries to Aquatechnics, Inc., to monitor bacteria concentrations both in the bay and within the hatcheries. In this manner, we will obtain a clear picture of the physical parameters affecting larval survival and growth in each location.

Conduct bioassays to assess the effectiveness of seawater treatment systems

Small scale bioassays will be conducted at each hatchery, as well as extensive bioassays to be conducted by Dr. Chris Langdon at Oregon State University's Hatfield Marine Science Center. These small scale experiments will allow for replication of cultures, and should provide direction for continued research efforts in 2009. However, small scale experiments are not always predictive of results in a commercial hatchery setting. Small scale experiments were run continuously throughout the spring and summer at Whiskey Creek, and clearly demonstrated that the stresses experienced in a 6000 gallon tank cannot always be reproduced in small, 10L containers. As a result, research in the fall of 2008 was conducted in the hatchery setting, which required considerable expenditures for heating of seawater, algal food production, and labor to maintain experiments. Although the costs of large scale bioassays are quite high, it is imperative that these experiments continue in the 2009 growing season, to fully assess the effectiveness of commercial scale water treatment systems.

#### BUDGET JUSTIFICATION

In 2009, a portion of Whiskey Creek Shellfish Hatchery will be dedicated to conducting commercial scale

bioassays, which will significantly impede commercial production efforts. However, these experiments are a crucial component of efforts to solve problems facing the oyster industry at large. Funds are therefore requested for personnel to conduct these experiments, including \$36,000 for the research coordinator and \$21,600 to support a research technician.

Taylor shellfish hatchery is currently conducting commercial-scale bioassays and intends to dedicate a sizable portion of their production capacity to understand and solve problems facing the hatcheries in 2009. Funds are therefore requested to support this work, including \$36,000 for the research coordinator and \$21,600 for a research technician. Taylor Shellfish will be matching \$38,400 to support the labor in this research effort.

Heating seawater for experimental purposes is quite expensive (\$50-75 in diesel costs per tank), and \$5000/month is requested to offset these costs to Whiskey Creek Shellfish Hatchery. Whiskey Creek has already absorbed most of the capital expenditures for seawater treatment systems, leaving approximately \$15,000 in new equipment to be purchased in this budget proposal. However, Taylor Shellfish Hatchery requires a major retrofit in term of water treatment since they don't have any of the seawater treatment systems already installed by Whiskey Creek Shellfish Hatchery. The capital expenditure funds requested for this purpose are \$64,650 and should cover the cost of hatchery retro-fitting and installation of monitoring systems.

Much of the remaining capital expenditures will go toward monitoring equipment and processing of seawater samples by independent labs. These expenditures, although substantial, offer the industry's best chance to fully understand the water quality issues facing our coastal bays and estuaries, and are critical to the success of this project. Matching funds will be supplied from the individual hatchery owners, in the form of labor, materials, and supplies to conduct and carry out commercial scale bioassays. Based on typical operating costs at Whiskey Creek Shellfish Hatchery, approximately \$5000/month will be spent in support of research activities in 2009, in the form of algal food production, labor, and electricity to power equipment. This represents a 1:1 matching of the funds requested to heat seawater in commercial scale bioassays. Taylor Shellfish hatchery is not requesting any diesel or "running" money and will dedicate approximately \$48,000 in matching fund for culture space and associated infrastructures (diesel, algae, water supply, electricity, etc.).

In addition, Whiskey Creek Shellfish Hatchery has spent approximately \$200,000 in capital expenditures for enhanced seawater treatment systems. The infrastructure established through installation of these systems will facilitate research efforts in 2009, and greatly expedite efforts to improve survival and growth of oyster larvae.

Matching funds will also be provided by the Molluscan Broodstock Program (MBP) at Oregon State University that will continue bioassays to improve seawater quality, and continue their in-kind contributions to the hatchery in the form of equipment and supplies. It is estimated that MBP will invest \$100,000 of research funding into this project, pending approval of their current budget request for the 2009 fiscal year. In-kind contributions from the Nature Conservancy will also support research activities at Whiskey Creek, in the form of labor and supplies totaling approximately \$20,000.

**APPENDIX 1: Part A****DR. BENOIT EUDELIN - PRODUCTION MANAGER, RESEARCH AND DEV. - TAYLOR RESOURCES**

SALARIES AND WAGES	Monthly	%	Number	Requested	Match	
	Stipend	Operation	months	funds	funds	
PI	\$5,000	60	12	36,000.00	24,000	
Technician	\$3,000	60	12	21,600.00	14,400	
<b>A. TOTAL SALARIES AND WAGES</b>				<b>\$57,600</b>	<b>\$38,400</b>	
<b>B. TAXES AND BENEFITS</b>				<b>\$11,112</b>	<b>\$8,808</b>	
<b>PERMANENT EQUIPMENT</b>			<b>Unit Price</b>	<b>Number</b>	<b>Requested</b>	<b>Match</b>
Gas saturometer			\$2,500	1	\$2,500.00	
Protein skimmer			\$6,000	2	\$12,000.00	
Gould Pumps			\$2,500	2	\$5,000.00	
Biofilter			\$2,500	2	\$5,000.00	
YSI photometer (chemical analysis)			\$2,550	1	\$2,550.00	
Counter flow degassing column			\$1,800	1	\$1,800.00	
Barben pH controller/injection system			\$3,800	2	\$7,600.00	
Barben ORP controller/injection system			\$3,800	1	\$3,800.00	
YSI 5200 monitoring system			\$1,695	4	\$6,780.00	
YSI multi-parameter probes			\$1,500	4	\$6,000.00	
YSI data logger (in the bay monitoring)			\$4,850	1	\$4,850.00	
Extech DO/pH kit			\$400	1	\$400.00	
UV sterilization Unit			\$1,800	1	\$1,800.00	
Ozone Generator			\$4,000	1	\$4,000.00	
Activated carbon filter (+media)			\$570	1	\$570.00	
Laptop computer (Panasonic Toughbook )			\$5,500	1		\$5,500
<b>C. TOTAL PERMANENT EQUIPMENT</b>					<b>\$64,650.00</b>	<b>\$5,500</b>
<b>EXPENDABLE SUPPLIES AND EQUIPMENT</b>						
Plumbing supplies				\$3,000.00		
Electrical supplies				\$1,000.00	\$2,000	
YSI reagents				\$3,000.00	\$1,000	
Laboratory supplies				\$1,000.00	\$1,000	
<b>D. TOTAL EXPENDABLE SUPPLIES AND EQUIPMENT</b>						<b>\$8,000.00</b>
<b>OTHER COST</b>						
Consulting fees				\$20,000.00		
Sample analysis						
Chemistry				\$10,000.00		
Bacteriology				\$10,000.00		
Electrician fees				\$1,000.00	\$1,000	
Culture space, infrastructures (diesel, water, algae...)					\$48,000	
<b>E. TOTAL OTHER COST</b>				<b>\$41,000.00</b>	<b>\$49,000</b>	
<b>F. TOTAL REQUESTED for Part A</b>				<b>\$182,362.00</b>	<b>\$105,708</b>	

**Emergency plan to save oyster production on the West Coast**

**APPENDIX 1: Part B****ALAN BARTON - RESEARCH MANAGER - WHISKEY CREEK SHELLFISH**

SALARIES AND WAGES	Monthly Stipend	% Operation	Number months	Requested funds	Matching funds
PI	\$3,750.00	80	12	36,000.00	9,000
Technician	\$3,000.00	60	12	21,600.00	14,400
<b>A. TOTAL SALARIES AND WAGES</b>				<b>\$57,600.00</b>	<b>\$23,400</b>

**B. TAXES AND BENEFITS** \$15,412.00

PERMANENT EQUIPMENT	Unit Price	Number	Requested	Matching
Gas Saturometer	2500	1	\$2,500.00	
2 HP pump	1000	1	\$1,000.00	
Activated carbon Filtration	2000	1	\$2,000.00	
YSI photometer (chemical analysis)	2550	1	\$2,550.00	
Counter flow degassing column	1800	1	\$1,800.00	
Barben pH controller/injection system	3800	2	\$7,600.00	
Barben ORP controller/injection system	3800	1	\$3,800.00	
YSI 5200 monitoring system	1695	2	\$3,390.00	
YSI multi-parameter probes	1500	2	\$3,000.00	
YSI data logger (in the bay monitoring)	4850	1	\$4,850.00	
Extech DO/pH kit	400	1	\$400.00	
Crushed coral filtration	3000	1	\$3,000.00	
Ozone generator	3000	1	\$3,000.00	
Laptop computer (Panasonic Toughbook)	5500	1	\$5,500.00	
Protein Skimmers	6000	2		\$12,000
Pumps	3000	4		\$12,000
Air Compressors	700	2		\$1,400
Bioreactors	5800	2		\$11,600
Flow control valves	3000	2		\$6,000
<b>C. TOTAL PERMANENT EQUIPMENT</b>			<b>\$44,390.00</b>	<b>\$43,000</b>

**EXPENDABLE SUPPLIES AND EQUIPMENT**

Seawater heating \$ for commercial - scale bioassays (\$5,000/mo)		\$60,000.00	\$60,000
Plumbing supplies		\$3,000.00	\$20,000
Electrical supplies		\$1,000.00	\$1,000
YSI reagents		\$3,000.00	\$500
Laboratory supplies		\$1,000.00	\$2,500
<b>D. TOTL EXPENDABLE SUPPLIES AND EQUIPMENT</b>			<b>\$68,000.00 \$84,000</b>

**OTHER COSTS**

Consulting fees		\$20,000.00	
Sample Analysis			
Chemistry	\$10,000.00		
Bacteriology	\$10,000.00	\$10,000	
Electrician Fees		\$1,000.00	\$10,000
<b>E. TOTAL OTHER COST</b>		<b>\$41,000.00</b>	<b>\$20,000</b>

F. TOTAL REQUESTED for Part B	\$226,402.00	\$170,400
G. GRAND TOTAL for Parts A and B	\$408,764.00	\$276,108

## **APPENDIX 2**

Coast-wide monitoring of commercially important bays and estuaries in the Pacific Northwest

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AMOUNT REQUESTED: \$340,500

MATCHING FUNDS: \$117,000

It is the goal of this proposal to begin a rigorous sampling scheme spanning a network of commercially important bays in the Pacific Northwest. The strength of the Pacific Coast Shellfish Growers Association (PCSGA) will allow us to coordinate this effort, and with the advice and help of local researchers, a comprehensive data set will be accumulated in 2009. With this information in hand, we will be better equipped to determine possible solutions, identify further research and monitoring needs, and help drive sensible policy. Only through concerted efforts to tackle this problem immediately can rapid progress be made toward fully understanding the devastating water quality issues facing our natural environment.

### **BACKGROUND AND RATIONALE**

Shortage of seed oysters has already translated into layoffs and decreased production in the 2008 season, with no obvious signs of relief in sight. Observations in 2007 and 2008 indicate water quality problems extend far beyond hatcheries and nurseries, and have had a dramatic impact on the health of our bays and estuaries. Extensive records from Willapa Bay, Washington, have shown a marked decrease in larval concentrations from 2005-2008. This data shows not only a decline in the prevalence of Pacific oyster larvae, which forms the cornerstone of production in the bay, but also shows a dramatic decrease across all species of larvae normally present in summer months. Even with significant improvements in hatchery production, the lack of significant natural oyster larvae in Willapa Bay and areas of Puget Sound will decimate the oyster industry, and production levels are projected to, at best, drop by 50% based on current trends. Although a monitoring program will not solve the problems facing the oyster industry, it will help us define water quality problems in a rigorous manner, facilitate ongoing research to better understand the issues facing our coastal economy, and provide us with tools for designing solutions.

Although the absence of larvae in the natural environment is a clear and unequivocal indication of severe problems for shellfish growers, very little hard data exists to support these claims. To date, the majority of large scale oceanographic monitoring programs have focused on the off-shore and open-water environments,

with few direct observations in the bays and estuaries where shellfish are produced.

## **SCOPE OF THE PROJECT**

The scope of this program will be limited to monitoring of our estuarine environment, and to integrate this monitoring with other relevant field and on-site observations.

What will this accomplish?

1) This monitoring program should serve as a resource for individual researchers, and provide accurate water quality data and seawater samples in support of their research needs.

Essentially, the monitoring program will provide a backbone to facilitate important research, and use the resources of PCSGA growers and hatchery workers to assist in the ground-level work of collecting samples and getting them into the right hands. This will hopefully free up resources for researchers to focus on data analysis and experiments in the lab, and alleviate the pressures of data and sample collection in the field.

2) The monitoring program will standardize data collection, which will allow for direct comparisons between sites.

Identifying a single lab to process each type of water sample will ensure that a consistent methodology is applied, and improve direct comparisons of data collected across the entire sampling region. Removing measurement error from the data set will make it easier to understand differences across sites and identify important trends in the data set.

3) The program will enlist the expertise of the research community at large, by providing a comprehensive data set from within our commercially important bays and estuaries.

Recent research suggests that the large dead zone previously detected along the Oregon Coast did not form in 2008, which led to improved water quality on the continental shelf. However, problems in coastal bays were, if anything, dramatically worse this past year. By collecting direct observations within these bays, we will be able to provide the research community with accurate data sets with which data can be compared to determine if there are corollary trends or patterns. These data will be made available to a wide variety of researchers to foster enhanced understanding on issues and trends that may be relevant to the concerns of shellfish producers.

4) Ultimately, the data sets will be correlated with reports of survival and growth of oyster larvae and seed, both in hatcheries and in the natural environment, and allow us to determine specifically which environmental parameters are of most concern to shellfish in commercially important bays and estuaries. This will be a highly useful tool for growers, whether remotely setting hatchery produced seed or setting cultch to collect wild seed.

This portion of the monitoring program will rely on contributions from oyster hatcheries and growers across the Pacific Northwest. In Willapa Bay, plankton sampling occurs frequently in summer months, as growers prepare to catch naturally occurring oyster larvae in the water column. In addition, this portion of the monitoring program will utilize data from a large number of research programs currently ongoing in these coastal bays and a focused set of larval experiments in a hatchery located in Willapa Bay. Most notably, this effort will be coordinated with research being conducted in Washington State by the Pacific Shellfish Institute, and in Oregon with research conducted both by the Nature Conservancy and Oregon State University. It is hoped that once the basic framework of data collection is established, a large number of complementary research programs will also become involved in the project.

## **COMPONENTS OF THE MONITORING SCHEME**

1) Water chemistry (discrete sampling):

Surface and subsurface samples collected at each site will be sent to the Burke Hales lab at OSU. This facility will provide accurate measurements of: Total Alkalinity (Alk), total dissolved inorganic carbon (DIC), pCO<sub>2</sub>, pH, nutrient levels (Ammonia, Nitrate, Phosphate, Sulfate), and a variety of potentially

## **Emergency plan to save oyster production on the West Coast**

important trace elements (Calcium, Strontium, Magnesium, etc.). At a minimum, samples will be collected weekly, with one sample taken at low tide, and another at high tide.

2) Water chemistry (continuous sampling):

Data loggers will be deployed at a fixed bottom elevation or water depth at each site to continuously monitor pH, temperature, salinity, dissolved oxygen, and ORP. These instruments will record hourly measurements of the above parameters. A large amount of data will be collected in this aspect of the monitoring program, and a biologist/data manager will be appointed to maintain these instruments, upload information, and manage the resulting data set.

3) Monitoring of *Vibrio* bacteria, total bacteria, and other potential pathogens:

Samples will be collected once per week, once at high tide and again at low tide, and sent to Dr. Ralph Elston at Aquatechnics lab for analysis. These tests will provide counts of total bacteria present in the sample, total counts of *Vibrio* species, and utilize a polyclonal antibody assay to test for the presence of pathogenic *vibrios* such as *Vibrio tubiashii*.

Periodic samples will also be made available on request to other labs investigating the effects of *Vibrio tubiashii* on oyster larvae, including labs operated by Dr. Carolyn Friedman at the University of Washington, and Dr. Claudia Hase at Oregon State University. These labs will test for the presence of other potential pathogens and stressors in these seawater samples, and refine techniques to rapidly quantify pathogenic bacteria in hatchery settings.

4) Assess condition of broodstock, spawning events and fertilization rates

During spawning season, broodstock samples will be collected from three sites in Willapa Bay every two weeks and histologies performed to determine the fecundity of mature animals. This data will be compared to historical condition indices to determine if there are trends in reproductive rates, and also be compared to other monitoring data to be collected to determine possible correlations between conditions and reproductive capability.

5) Comparison with larval performance using hatchery data & plankton tows:

Weekly scoring of larval performance will be conducted (using a scale of 1-10). Plankton tows are already available from Willapa Bay on a weekly basis. Plankton nets will be purchased and provided to sites that currently have no hatchery data or ongoing plankton sampling program, but that can supply the personnel and other equipment required to collect samples. Personnel at each site will also be trained to identify and innumerate collected larvae.

When larvae are first detected in the plankton of Willapa Bay, broodstock oysters will be collected and spawned in a commercial hatchery located in the Bay (Weigardt Bros. Inc.). The larvae will be reared in 10 µm-filtered seawater pumped from the bay and fed on rations of cultured algae, using standard hatchery protocols. Larvae will be raised at both ambient temperature and at 25 0C. The growth, survival and setting of the hatchery-reared larvae will be compared with that of larvae in the plankton. In this way, it will be possible to determine if food availability or water temperature or both are limiting factors in determining larval set.

6) Larvae and/or broodstock will be sent to the Marine Invasions Research Lab at the Smithsonian Environmental Research Center in Edgewater Maryland where Dr. A. Whitman Miller will run experiments on how oyster larvae respond to continuously elevated levels of CO<sub>2</sub> from 72 hrs post-fertilization to 30 days. Dr. Miller is currently engaged in studies involving *Crassostrea virginica* and *Crassostrea ariakensis*

oysters to determine the effect of CaCO<sub>3</sub> saturation on larval development. He will run trials in his lab to determine the effect of a range of Ph and *Vibrio tubiashii* levels on *Crassostrea gigas* (the most prevalent oyster produced on the West Coast) larval development.

7) Water samples will also be used to sample for the presence of harmful algal blooms or to determine shifts in algal compositions when natural oyster larvae populations are present.

### **SITE LOCATIONS AND PARTICIPANTS**

Included is a preliminary list of growers/researchers who have expressed an interest in participating in the monitoring program. Specific participants will be chosen from among this list when funding becomes available.

Humboldt Bay (Arcata), CA: Kuiper Mariculture

Yaquina Bay, OR: Molluscan Broodstock Program

Netarts Bay, OR: Whiskey Creek Shellfish Hatchery

Tillamook Bay OR: Mark Weigardt

Willapa Bay, WA: Northern Oyster, Stony Point Oyster, Coast  
(multiple sites) Seafoods, Wiegardt Bros., Taylor Shellfish

Puget Sound, WA: (3 sites) Taylor Shellfish, Lummi Hatchery, Coast Seafoods

A sampling grid at three sites used for seed collection will be established in Willapa Bay, through coordination with existing monitoring programs in the bay, Puget Sound sites (3 total) will be in waters adjacent to the Coast Seafoods and Taylor Shellfish hatcheries in Hood Canal, and the Lummi hatchery in north Puget Sound.

\*British Columbia, Canada. David McCallum of the BC Shellfish Growers Association has expressed an interest in participating in the program. Any participation from BC growers will be funded independently through the BC Shellfish Grower's Association, and would not make use of funds provided via this proposal.

### **BUDGET JUSTIFICATION**

This program represents a comprehensive approach to water quality monitoring, and is intended to monitor a large suite of seawater parameters. This approach will allow researchers to understand interactions between a wide variety of physical and chemical parameters, and understand their combined effects on the survival and growth of oyster larvae and seed. This approach also identifies individual labs to collect and interpret data, and will provide a consistent data set with a minimum of measurement error.

Burke Hales, a chemical oceanographer at Oregon State University's College of Ocean and Atmospheric Sciences, has been identified to process water chemistry samples throughout the 2009 growing season. Hale's lab specializes in accurate chemical analyses of seawater samples, and can provide high resolution data through a consistent and well-established approach. The lab estimates that sample analysis will cost approximately \$50/sample. Two weekly samples from each of the ten sites described in this program will produce a total annual cost of \$52,000 for chemical analysis of seawater samples.

Dr. Ralph Elston of AquaTechnics, Inc. has worked extensively with the Pacific Coast shellfish industry for over a decade, and has developed rigorous protocols to monitor total bacteria and *Vibrio tubiashii* concentrations in seawater, and has developed sophisticated tools to monitor the pathogenicity of *Vibrio* bacteria. Moreover, Dr. Elston has conducted extensive routine monitoring for the shellfish industry in

### **Emergency plan to save oyster production on the West Coast**

recent years, and is able to provide reliable results in a timely fashion. Therefore, AquaTechnics has been identified to process seawater samples for bacterial enumeration, at a cost of \$50/sample. The total annual cost for two samples per week from each of the ten sites will total \$52,000. Dr. Elston will also perform the histologies on adult oysters to determine fecundity, at \$12 per sample for 600 samples (30 oysters samples over 20 sampling event during typical spawning season in the spring and summer).

Dr. Whitman Miller will perform two 30 day laboratory experiments to determine effects on oyster larval to varying levels of *Vibrio tubiashii* and Ph levels for a total cost of \$45,000 which includes Dr. Miller's and a technical assistant's time.

The Pacific Shellfish Institute will assess preserved plankton samples to determine the taxa and abundance of algal populations at an average cost of about \$30 per sample.

The YSI instrumentation to be used in this monitoring program has been selected through consultation with researchers across the Pacific Northwest, with the goal of producing a reliable data set that will be relevant to the scientific community at large. YSI instruments are well respected by the research community, and have a long history of providing reliable data in coastal environments. In addition, the YSI 6600 EDS Sondes listed in the attached budget are currently being used by the Pacific Shellfish Institute and the University of Washington in monitoring programs already underway in both Willapa Bay and Puget Sound. Choosing identical instrumentation will facilitate sharing and comparison of data, and ensure consistency across the resulting data set. The total cost for the 12 data loggers requested in this proposal is \$88,500, and allows for frequent maintenance and recalibration of units through the inclusion of two additional loggers.

To manage the large amount of data collected in this monitoring program, a biologist/data manager will be identified, to be funded at a total cost of \$50,000. The person will be responsible for deployment, maintenance and calibration of instruments, and will coordinate shipments of samples from all growers participating in the program. He/she will assist in field collection coordination, and provide training and proper QA/QC protocols for volunteer participants to ensure uniformity and accuracy in collection methods and larval/plankton identification. In addition, the biologist/data manager will likely have shared responsibility for maintenance and operation of the Willapa hatchery facility.

Remaining funds requested in this proposal will pay for the weekly shipping of samples from each site to the labs specified above, and periodic shipping of loggers to the data manager for routine maintenance and uploading of data (\$24,000) An additional \$25,000 is included for miscellaneous supplies and materials, including plankton nets for growers interested in monitoring larval concentrations in local bays, instrument deployment and maintenance supplies, sampling materials, and general travel costs for the biologist/data manager to access the field sites.

Matching funds for this program will be supplied by the Pacific Coast Shellfish Growers Association (PCSGA), in the form of time, materials, and ship or vessel time required collecting samples for the program. The strength of PCSGA offers a unique opportunity to build an extensive and reliable data set, and the interest and concern of Pacific Coast shellfish growers provide a motivated workforce already equipped with appropriate shallow water vessels at no additional cost to the monitoring program. Moreover, the coordination between PCSGA and the project team will ensure samples are collected following prescribed protocols and with sufficient frequency and geographic coverage to allow the team members to determine important trends in the resulting data set.

APPENDIX 2		Number	Requested	Matching
SALARIES AND WAGES		months	funds	funds
Data Manager		12	\$38,400.00	
Shellfish companies/sampling/monitoring		12	\$40,000	
A. TOTAL SALARIES AND WAGES			\$38,400.00	\$40,000
B. TAXES and BENEFITS			\$12,900.00	
PERMANENT EQUIPMENT		Unit Price	Number	Requested
YSI 6600 EDS Data loggers w/ optical DO		7375	12	\$88,500.00
B. TOTAL PERMANENT EQUIPMENT				\$88,500.00
EXPENDABLE SUPPLIES AND EQUIPMENT				
Shipping supplies			\$8,000.00	
Standard solution, tools to maintain instrumentation			\$10,000.00	
Plankton nets			\$5,000.00	
Laboratory Supplies			\$2,000.00	\$5000.00
Vessels				\$30,000
C. TOTAL EXPENDABLE SUPPLIES AND EQUIPMENT			\$25,000.00	
OTHER COSTS				
Chemical analysis of seawater samples (Burke Hales, OSU)			\$52,000.00	
Microbial analysis of seawater samples (Aquatechnics, Inc.)			\$52,000.00	
Broodstock histologies (Aquatechnics, Inc.)			\$8,400.00	
Harmful Algal Bloom sampling (Pacific Shellfish Institute)			\$7,200.00	
V.t. and Ph effects on larvae - lab tests (Dr. Whitman Miller, Smithsonian)				\$45,000.00
Shipping costs			\$24,000.00	\$2,000
D. TOTAL OTHER COST			\$188,600.00	
E. GRAND TOTAL REQUESTED			\$340,500.00	\$117,000

## Appendix 3

### **RAPID SCREENING OF OYSTER GERMPLASM RESOURCES TO IDENTIFY FAMILIES RESISTANT TO ADVERSE ENVIRONMENTAL CONDITIONS**

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Chris Langdon, Ph.D.  
Professor of Fisheries  
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AMOUNT REQUESTED: \$131,228  
MATCHING: \$61,500

#### **BACKGROUND AND RATIONALE**

While improvements to hatchery seawater treatment systems can potentially mitigate the impacts of poor water quality and vibriosis on commercial-scale larval culture, we also have qualitative, anecdotal evidence that using genetically superior breeding stock can have an immediate and profound impact on larval and juvenile survival and growth under these adverse environmental conditions. The overall production of oyster seed at the Whiskey Creek shellfish hatchery was drastically reduced in 2008, but one specific batch of larvae experienced much lower mortality and higher growth in both the hatchery and the subsequent “nursery” phase of culture (which typically occurs either in land-based systems supplied with untreated seawater or in the natural environment). This group was later determined to be derived from genetically improved breeding stock obtained from the Molluscan Broodstock Program (MBP), an ongoing selective breeding program conducted at Oregon State University’s (OSU) Hatfield Marine Science Center (HMSC) in Newport, Oregon and directed by Chris Langdon [1]. Because this group was raised “side-by-side” with batches derived from other parents, the observed difference most likely has a genetic basis.

Prior to the current hatchery crisis, MBP’s standard selective breeding protocols have not included selection on larval traits, but in 2004 and 2005, before the onset of crisis-level mortalities in commercial hatcheries, the research-scale hatchery at HMSC experienced unusually low larval and spat survival. These problems were partially mitigated by an extensive reconfiguration of the hatchery water treatment systems, but during this period, MBP reared several cohorts consisting of approximately 50 families each that experienced severe, but not complete, larval mortality. In all likelihood, these cohorts have been subjected to intense “natural” selection for larval resistance to some of the same stresses that are now causing such severe

problems in commercial hatcheries. Based on qualitative hatchery observations during the larval phase of culture and quantitative data collected during the nursery phase (Fig.1), it is clear that some genetically discrete families within these cohorts showed a much higher survival rate than others, further supporting the idea that there is a genetic basis to early survival under adverse conditions.

Unfortunately, we have no simple and reliable means of predicting which crosses among these families will produce high larval and juvenile survival because we do not fully understand the genetic mechanisms responsible. In addition, we cannot be 100% certain that these cohorts of larvae were exposed to the same stresses that are currently impacting commercial hatcheries or that these stresses have similar impacts in large (e.g. 10,000 gallon) commercial-scale tanks as in the small (2-5 gallon) research-scale tanks used to rear MBP families. Thus, while we have strong reasons to believe that our current broodstock inventory contains families that are more resistant, and potentially much more resistant to the adverse conditions currently impacting commercial oyster hatcheries, the only way to quickly identify those families is to screen our current germplasm holdings in an appropriate manner.

We propose to accomplish this through a collaborative effort between MBP/OSU, USDA-ARS and commercial hatcheries that ensures that we will not only focus on the appropriate stressors, but also that we conduct our trials at the appropriate scales by performing most of the experimental portions of the work in commercial facilities rather than in research facilities that may or may not experience exactly the same problems as production-scale facilities. In addition, because this effort will focus on families for which we currently have large numbers of breeding-age animals on-hand, once we identify superior families, they can be made available to industry immediately rather than after the more typical delay of 2-3 years to raise sufficient numbers of reproductively mature breeders.

Specifically, we propose a two-phased procedure for characterizing currently available breeding families that uses a mixed-family screen conducted within commercial hatchery facilities to simultaneously evaluate a large number of genetic families followed by laboratory bacterial challenges on only the most promising families to ensure that they are resistant to the most important bacterial pathogen identified to date, *Vibrio tubiashii*.

## **EXPERIMENTAL APPROACH**

### **Phase 1: Mixed family screen under commercial conditions**

The first phase of this screening effort consists of a “mixed family” approach similar to the so-called “walk back selection” [2, 3]; implemented within commercial hatcheries. This approach uses extensive pooling of genetically distinct families to simultaneously test large genetic arrays under commercial conditions. Due to the costs involved in producing these arrays of families, MBP will begin the 2009 spawning season in “standby mode,” by holding representatives from all potential families in reproductive condition and waiting until mortality problems arise in commercial hatcheries to initiate the mixed family experiment. This will ensure that we do not inappropriately and wastefully invest effort or funding conducting these costly and complicated experiments at a time when the hatcheries are not experiencing problems while also ensuring that we will be ready to respond immediately by producing the necessary controlled spawns within a few days when problems do arise.

In addition, when commercial hatcheries attempt to resume full-scale production in 2009, they will contribute to this effort by dedicating a small number of their large larval rearing tanks to rearing “sentinel groups” of larvae in the minimally treated seawater that was standard operating procedure before the current problems emerged. The use of these sentinel groups will allow the early detection of compromised seawater

quality, and as soon as mortalities are observed, MBP personnel can create a large number of genetically distinct crosses (>100) among the families that qualitative hatchery observations indicate had the best larval and juvenile survival in the MBP hatchery in 2004 and 2005 (cohorts 20 & 21). These crosses will be constructed using controlled fertilization procedures followed by mixing of the families 24 hours post-fertilization and subsequent larval rearing under the actual conditions that are causing the problem.

The mixed cultures will be closely monitored for mortality and we will collect samples of the larvae that survive and successfully complete metamorphosis in these mixed family tanks by adding settling substrate to the tanks on a daily basis after we first observe metamorphically competent larvae in the tanks. This procedure will allow us to identify individuals that not only survive as larvae, but to also collect data on development time, and most importantly, we can be confident that we are collecting individuals that go on to produce viable seed oysters for commercial grow-out. In addition, a large sample of competent larvae will be sent to a remote setting facility in Willapa Bay and set on shell cultch following standard commercial-scale procedures.

Once these successful juveniles have been collected, the USDA-ARS Shellfish Genetics Laboratory will use microsatellite DNA genetic markers to assign individuals to their parents within a short period of time. While the molecular genetic technology to achieve this objective is complicated, the principle is actually quite simple. Microsatellite DNA markers are highly variable within populations, with some specific markers having 50 or more variant forms. Because these variations are reliably transmitted from parent to offspring according to Mendelian genetic rules, if multiple marker genotypes are available from both parents and offspring, it is a relatively simple matter to determine the exact parentage of single individuals derived from mixtures of families using any number of existing computer programs specifically developed for this purpose such as WHICHPARENT [Banks, unpublished], PROBMAX [4] and CERVUS [5]. Over 100 microsatellite markers have been developed for Pacific oysters [6-14], and nearly all of these have been optimized and are available for use in the USDA Shellfish Genetics laboratory. Performing these parentage analyses is a standard technique in many genetics labs, including the USDA-ARS Shellfish Genetics Program, which has also developed new computer programs to optimize the selection of markers [15], and we anticipate that we can accurately assign offspring to parents using 10-20 markers.

#### Phase 2: Separate-family laboratory challenges with *Vibrio tubiashii*

As pointed out in the main portion of this proposal, the mortality problems currently plaguing shellfish hatcheries in the Pacific Northwest are most likely caused by several interacting factors that are currently poorly understood. Even so, previous experience indicates that *Vibrio tubiashii* (*Vt*), a highly virulent bacterial pathogen likely plays a major and possibly dominant role. While this pathogen may well be a contributor to mortality in the Phase 1 mixed family experiment, given the unpredictable nature of *Vt* outbreaks and the severity of their impacts, it seems worthwhile to ensure that any breeding stock provided to the oyster culture industry is as resistant as possible to *Vt*. Therefore, we propose to subject the top 10-20 families from the mixed family screening above to separate family controlled laboratory challenges using highly virulent cultured *Vt* as both larvae and juvenile “spat.”

##### Larval Challenges

We will assay resistance to *Vibrio tubiashii* (*Vt*) pathogenicity using the most pathogenic isolate currently available of *Vt* in 1000 ml plastic beakers at room temperature using ~10-day old larvae produced by re-creating the top-performing crosses from the mixed family experiment and a mixed control group. We will begin each challenge by adding 2000 healthy larvae to family-specific beakers and then inoculating the beakers with cultured and washed *Vt* bacteria. All beakers will be checked every 2-3 hours depending on the rate of mortality observed, and dead larvae will be collected and preserved in ethanol at each check. Surviving and dead larvae will then be counted to identify the families with the highest larval survival

## Spat Challenges

We will perform spat challenges using the same high-surviving families and controls. These experiments, however, will not use group challenges in large vessels as above, but rather 120 individual spat held in 24-well plates. We will first distribute the spat into their individual wells, and then inoculate all wells with cultured bacteria. Plates will be checked every 2-4 hours depending on the rate of mortality observed, and at each check, dead or moribund individuals will be recorded.

## EXPECTED RESULTS

Insofar as this work is proposed as an emergency measure to address an emerging and poorly understood crisis, it is difficult to precisely predict the outcomes. Nonetheless, our expectation is that once we identify the specific families that are most capable of surviving as larvae and successfully metamorphosing into adults under adverse environmental conditions, commercial hatcheries will be able to re-create these families using the brothers and sisters of the parents of survivors as their breeding stock, and immediately generate a high-surviving genetic stock that would help restore commercial-scale seed production. Large quantities of broodstock oysters (>500) from each of these parental families are available from MBP's repository in Netarts Bay, Oregon for this purpose.

## BUDGET JUSTIFICATION

The mixed family approach will require MBP personnel to produce the arrays of families within commercial hatcheries. This procedure requires meticulous and time-consuming procedures to ensure that controlled fertilizations produce the desired families and to equalize the numbers of larvae contributed by each family to the test mixtures. It is unrealistic to expect commercial hatchery operators to perform these specialized procedures. In addition, the laboratory bacterial challenges will require strict application of quarantine conditions to prevent the spread of *Vibrio tubiashii* in the MBP hatchery, and coastal waters. MBP has established the necessary quarantine protocols and has the use of a larval and spat rearing facilities where all effluent is treated by chlorination before discharge.

We have requested funds to support two weeks of time for a post-doc to supervise the project. In addition, we request funding to support a total of 4 months of technician time. \$2000 is requested for supplies and \$1500 for travel as it will be necessary for personnel to travel to work at commercial hatcheries. We have also conditionally budgeted for OSU indirect costs of 46.2%.

In addition, determining the parentage of surviving spat will require personnel and consumable supplies for the USDA-ARS Shellfish Genetics Laboratory. We anticipate that genotyping approximately three thousand survivors, from both the hatchery set and remote-setting experiments, and that 10-20 microsatellite DNA markers will be required for accurate parentage assignment given the large numbers of parents that will contribute to the family mixtures. Our cost analyses indicate that DNA extraction requires almost exactly \$1.00/sample in consumable supplies using "home made" purification columns or \$3,000 for 3,000 samples. In addition, PCR amplification and automated electrophoresis of one microsatellite DNA marker from one sample requires \$0.36 in consumable supplies and \$0.50 in fees for use of shared equipment or \$77,400 to type 6,000 samples at 15 markers. Finally, the USDA Shellfish Genetics Laboratory is completely equipped for this effort, but currently employs only one full-time technician with a wide range of other responsibilities at this time. Including the time required to collect and preserve tissue samples, we have budgeted for two months of technician time. We have also conditionally budgeted for USDA-ARS indirect costs of 10%.

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APPENDIX 3: Part A		
Mark Camara, USDA-ARS	Requested	Matching
SALARIES AND WAGES	Funds	funds
Res. Asst. 2 FTE	\$7,750.00	
Shellfish companies: remote setting experiments		\$10,000.00
A. TOTAL SALARIES AND WAGES	\$7,750.00	\$10,000.00
B. TAXES and BENEFITS	\$2,326.00	
EXPENDABLE SUPPLIES AND EQUIPMENT		
Supplies	\$83,400.00	\$10,000.00
Vessels	\$10,000.00	
C. TOTAL EXPENDABLE SUPPLIES AND EQUIPMENT		\$83,400.00
\$20,000.00		
D. INDIRECT COST	\$9,348.00	
E. TOTAL REQUESTED for PART A	\$102,824.00	\$30,000.00
APPENDIX 3: Part B		
Chris Langdon, OSU-MBP	Requested	Matching
SALARIES AND WAGES	funds	funds
PI .042 fte @ 0.5 mo	\$ 2,222.00	
Res Asst. 1 fte@ 2 mo	\$5,840.00	
Temp 0.167 fte @ 2 mo.	\$ 2,400.00	
Shellfish companies: remote setting experiments	\$10,000.00	
A. TOTAL SALARIES AND WAGES	\$10,462.00	\$10,000.00
B. TAXES and BENEFITS	\$5,466.00	
EXPENDABLE SUPPLIES AND EQUIPMENT		
Supplies	\$2,000.00	\$10,000.00
Vessels	\$10,000.00	
C. TOTAL EXPENDABLE SUPPLIES AND EQUIPMENT	\$2,000.00	\$20,000.00
D. TRAVEL	\$1,500.00	\$1,500.00
D. INDIRECT COST	\$8,976.00	
E. TOTAL REQUESTED For Part B	\$28,404.00	\$31,500.00
F. GRAND TOTAL Parts A and B	\$131,228.00	\$61,500.00

## Appendix 4

### DEVELOPMENT OF A RAPID 'DIPSTICK' ASSAY FOR ON-SITE DETECTION OF *VIBRIO TUBIASHII* IN COMMERCIAL SHELLFISH HATCHERIES

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AMOUNT REQUESTED: \$ 79,633

#### BACKGROUND AND RATIONALE

The primary goal of our research is to stabilize and increase shellfish hatchery and nursery production by reducing the occurrence of larval and juvenile mortalities through the development of a rapid, specific, sensitive, and quantitative detection method, which will be a useful on-site tool in identifying pathogen and toxin contamination in bivalve rearing systems (1). We have previously shown that a secreted metalloprotease is the critical factor for the toxicity of *Vibrio tubiashii* supernatants (toxic molecules secreted by the pathogen) on Pacific oyster larvae (2) and have purified the metalloprotease using molecular methods (His-tag) (3). Our specific aims are to (i) utilize our purified metalloprotease from *V. tubiashii* to develop monoclonal antibodies and (ii) use such antibodies to develop a lateral flow dipstick detection assays. Our rationale for this project is that by providing a rapid, specific and reliable detection method for the *V. tubiashii* metalloprotease, that is then used in hatchery and nursery settings, early detection of pathogenic vibrios and their toxins at these production sites will initiate management and prevent outbreaks of the disease.

In spite of the economic consequences of *V. tubiashii* in the cultivation of larval and juvenile bivalves in shellfish hatcheries, specific tests to detect the secreted metalloprotease toxin at lethal or sub-lethal levels are lacking. This gap prevents the application and further refinement of targeted intervention strategies to effectively eliminate this pathogen and its toxins from shellfish hatcheries. Our research approach is expected to yield the following outcomes:

From our research we anticipate providing the shellfish industry with useful tools to help them prevent bacterial diseases in their hatcheries. Accurate detection of the toxic protease will allow the hatchery to better adjust water systems and operations management to maximize survivability, growth and condition of larval and juvenile bivalves. It is not cost effective to proceed with culture of animal groups if it is uncertain whether they will survive or continue to develop properly in the presence of sub-lethal concentrations of *V. tubiashii* protease. In addition, the tool we propose to develop will support a much broader scale of research on the effects of the pathogenic cosmopolitan *V. tubiashii* on natural populations of marine species.

Objective #1: Develop monoclonal antibodies targeting the *V. tubiashii* secreted metalloprotease. Our previous work provides the key for the successful development of monoclonal antibodies - the use of our previously purified His-tagged metalloprotease. We will cost-effectively contract the production of specific monoclonal antibody cell lines to a commercial laboratory specializing in this service, as is normally done

for such applications. The production of these monoclonal antibodies will be the basis for our development of the fieldable detection assays for the important toxigenic factor produced by virulent *V. tubiashii*.

**Rationale.** Immunoassays are used when an unknown concentration of an analyte within a sample needs to be quantified. Although several different types of immunoassays can be utilized for this, they all rely on the quality of the antibodies used. Typically, monoclonal, as opposed to polyclonal, antibodies are used because (i) the antibodies are of a single specificity and are all of identical structure because they are being manufactured by a single clone of plasma cells, (ii) they can be cost effectively produced in large quantity and (iii) they can be produced indefinitely by the cultured cell lines.

**Generation of monoclonal antibodies.** The process by which large quantities of antibodies that are targeted against a particular antigen can be produced by cultured cell lines is quite labor intensive and it is now commonplace to provide highly purified target protein to a specialized contract laboratory that has a protocol we have reviewed and approved. Once a monoclonal antibody is made, it can be used as a specific probe to detect the target protein that was used to induced its production. Several companies specialize in the production and initial testing of monoclonal antibodies and we have budgeted money for this work to be done by AbboMax, Inc. (San Jose, CA), a company with extensive experience in this type of service.

**Expected results and potential limitations.** We do not anticipate major problems for this part of the work. We have already purified substantial amounts of His-tagged metalloprotease from *E. coli* cells expressing the *V. tubiashii* vtpA gene that was enzymatically active (3). The commercial company will perform all of the key steps in the production of the monoclonal antibodies, including antigen injections into mice, hybridoma production, screening of the clones, purification of supernatants and preliminary ELISA evaluations using the recombinant protein. In the unlikely event that the purified protein is not sufficiently immunogenic in mice, synthetic peptides based on the metalloprotease amino acid sequence could be generated and used to generate monoclonal antibodies.

**Objective #2: Develop a lateral flow dipstick assays.** We will incorporate the monoclonal antibodies raised against the *V. tubiashii* metalloprotease to establish a sensitive and specific detection assay that can quantitatively measure metalloprotease levels in various marine sample types. We will use sets of antibodies to develop a quantitative lateral flow dipstick assay using an immunological sandwich-binding scheme for a fieldable assay.

**Rationale.** We will develop a lateral flow dipstick assay that can easily be used on site in a hatchery as a rapid diagnostic tool. Lateral flow dipstick assays are widely established technology for detection and diagnostic purposes ranging from home pregnancy tests to point-of-care malaria tests. If desired, the dipsticks can also be analyzed quantitatively by using an absorbance reader.

**Lateral flow dipstick development.**

The lateral flow assay technology provides a fast, reproducible, quantitative method for measuring antigen quantity and/or activity from a wide range of cellular material. Dipstick quantity assays utilize an immunological sandwich-binding scheme where two monoclonal antibodies recognize different epitopes on the same protein or enzyme complex. One antibody is embedded on a nitrocellulose membrane, while the other is conjugated to gold particles (Fig. 1). The top band is the positive control and underneath is the band for the target protein (Fig. 2). The intensity of the target band correlates directly with the protein levels present in the sample.

Figure 1: Detailed overview of antibody/antigen interactions.

Figure 2: Schematic representation of a lateral flow dipstick.

This type of lateral flow technology has been very successful in a variety of applications due to these advantages over other methods of quantitative antigen concentration evaluation: the assays are fast (30 to 120 minutes), easy to use, non-invasive, quantitative, and require little starting sample material.

**Assembly of dipstick cards.** The construction of dipsticks requires initial assembly of 6 cm x 30 cm cards, with each card using the following components: MIBA 010 Adhesive backing (30 cm length with 8 cm width cut to 6 cm); Millipore High Flow (HF) 120 nitrocellulose membrane (2.5 width and 100 m length cut in strips to ~32 cm), and Millipore cellulose fiber sample pads (CFSP, 20 cm x 30 cm, with 20 cm length cut to 3.7 cm). For each dipstick card, adhesive backing is removed from the MIBA card, the HF 120 membrane is aligned along the bottom edge of the MIBA backing, and the CFSP is aligned along the top edge of the MIBA backing, allowing for an overlap between the membrane and wick pad of 1 to 2 mm. A speedball roller is used to firmly adhere the CFSP to the adhesive backing.

**Antibody application and cutting dipstick cards.** Antibodies received from the Monoclonal Antibody Facility will be placed on ice and diluted with HEPES-Buffered Saline (HBS) and 0.02% sodium azide (preservative) to specific antibody concentrations to determine the optimal performance in the dipstick assays. Goat anti-mouse Ig-Fc antibodies will be applied to serve as a positive control.

The dipsticks will be stripped with antibodies using a simple slot dispenser apparatus (Immunetics Miniblitter Systems, Immunetics, MN). This device allows for the application of a precise volume of liquid reagent through a slot onto a membrane (as measured in  $\mu\text{l}$  reagent/mm membrane). A pre-assembled dipstick card is then aligned on the dispenser platform the slots will be used to apply 2 lines (one for each of the antibodies) onto the membrane portion of the card. The slots will be primed with buffer to ensure that the lines are clear of residue and 600  $\mu\text{l}$  of each monoclonal antibody will be applied and incubated overnight at 4°C. Once the dispensing is complete, the card is visually checked to determine that the monoclonal antibody lines are uniform, and the card is placed in a 37°C air incubator for 1 hour to dry the monoclonal antibody and further adhere the wick pad and membrane to the adhesive backing. The card is then placed in an auto-desiccator cabinet for a minimum of 24 hours. To cut the final strips, each dipstick card is placed on a platform and fed through while a blade cuts the card approximately every 4 mm. (The final dimensions of the dipstick are 4 mm width x 6 cm length). Each stick is visually inspected for anomalies, and then stored in plastic bags with silica packs. One dipstick card yields approximately 60-70 dipsticks.

**Preparation of gold colloid/monoclonal antibody conjugation.** 40-nm gold colloid will be purchased from Structure Probe, Inc., (West Chester, PA), brought from 4° C to room temperature and the pH will be raised from ~5 to 9 with 0.2 M K<sub>2</sub>CO<sub>3</sub>. A specific concentration of the monoclonal antibodies will be added to separate aliquots of the pH-ed gold colloid. The tubes will then immediately be inverted for 1-10 minutes. 10% bovine serum albumin (BSA, a blocking reagent) will be added and the tubes are again immediately inverted for 1-10 minutes. The tubes are then spun at 5,000 rcf at 4° C for 20 minutes, after which the supernatants are aspirated. The remaining gold/monoclonal antibody conjugation is re-suspended in passive gold diluent (a reagent comprised of phosphate buffer/BSA). The proper concentration of gold-conjugate is determined by optical density (OD): 700  $\mu\text{l}$  of sample is placed in a cuvette in a spectrophotometer reader. The sample is read and analyzed using a software program via determination of the optical density at the maximum peak displayed. Based upon this OD reading, each monoclonal antibody gold-conjugate is diluted with passive gold diluent in order to determine the optimal OD. At this point, both monoclonal

antibody gold-conjugates are combined in equal volumes, and specified amounts of sucrose and trehalose are added in order to maintain monoclonal antibody structure. After mixing well, 10 µl aliquots are pipetted into 96-well microplates, and then placed in a 37° C air incubator overnight. After 24 hours, the microplates are stored in desiccator cabinets.

Dipstick Assays. The procedure for dipstick sample absorption/washing/drying will be followed essentially as described previously (16). The general protocols for the lateral flow dipstick assays will be as follows.

Dilution series: A standard curve is established via use of a dilution series with a sample of known protein concentration. In the first well of a microplate, a volume of Buffer A (extraction buffer) is added to cell extract to bring the volume up to an equal amount of Buffer B (block). The next 7 wells contain an equal mix of Buffers A and B (each totaling half the volume of well 1). Half the volume of well 1 is transferred to well 2 and mixed. Half the volume of well 2 is transferred to well 3 and mixed. This procedure is continued until well 7, where, after sample from well 6 is added, an equal amount of the total diluted sample is removed. Well 8 serves as a blank (containing no sample, only an equal mix of Buffers A and B). 50 µl from each well of this dilution series is then transferred to microplate wells containing gold-conjugate, allowed to sit 5-7 minutes, and pipetted to mix.

Repeat series: In order to test several dipsticks with the same amount of sample to validate repeatability, a master mix is made consisting of Buffer A plus sample, and a matching volume of Buffer B. 50 µl of this sample is added to the desired number of gold-conjugate microplate wells, allowed to sit 5-7 minutes, and pipetted to mix. Dipsticks are then added (with the thin nitrocellulose end in the sample well) until all sample is absorbed from each well (~25 minutes). 30 to 60 µl of Buffer C (Wash Buffer) is then added to each well and allowed to wick up the dipstick (~17 minutes). After all Buffer C had been wicked from the microplate well, dipsticks are removed from the wells and dried at RT for 30 minutes or more, or in a 37° C air incubator for 10-15 minutes.

Measurement and Analysis of Gold-Conjugated Antibody Signal. The signal intensity of each dipstick is read using a Hamamatsu MS1000 Dipstick reader and ICA 1000M software. The absorbance of the gold-conjugate signal is measured by the reader as follows: the darker a signal band, the more light absorbed, and the greater assigned absorbance value. Absorbance readings are displayed as peak values and as a quantitative unit that is x1000 for ease of graph displays. Dilution series absorbance values are then plotted in an Excel graph with protein µg featured on the x axis and absorbance values on the y axis. The standard curve obtained can be narrowed to a best-fit linear range. Dipstick absorbance values from the “repeat series” are averaged, and standard deviation and % coefficient of variance are calculated via Excel.

Expected results and potential limitations. The assay development itself is straightforward and we do not anticipate major problems during these steps. Typically, monoclonal antibodies are highly specific (i.e., they recognize only one epitope on the antigen) and with sufficient screening, an antibody (to then be used as the capture antibody) that is highly specific for the *V. tubiashii* metalloprotease should be identified, thus alleviating any concerns about lack of specificity or cross reactivity. We do not anticipate any insurmountable problems, based on our preliminary work and knowledge of the structure and action of vibrio generated proteases.

Expected impacts. This project addresses a recent crisis affecting shellfish hatcheries in the United States and the project product will likely assist health management in shellfish hatcheries. Previous work by the Häse lab has shown that the extracellular metalloprotease secreted by *V. tubiashii* is highly toxic to Pacific oyster larvae (2). The development of a fieldable, rapid, reliable, sensitive and cost-effective early detection method for this key and widely occurring toxic protein would have an enormous impact on the effective

management of water quality in shellfish hatcheries and can be expected to ultimately help this entire industry to survive this challenge (1).

## REFERENCES

1. Elston, R. A., H. Hasegawa, K. L. Humphrey, I. K. Polyak, and C. C. Häse. Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and management. Dis. Aquat. Org., in press.
2. Hasegawa, H., E. J. Lind, M. A. Boin, and C. C. Häse. 2008. The extracellular metalloprotease of *Vibrio tubiashii* is a major virulence factor for Pacific oyster (*Crassostrea gigas*) larvae. Appl. Environ. Microbiol. 74: 4101–4110.
3. Hasegawa, H., D. N. Gharaibeh, E. J. Lind, and C. C. Häse. Virulence of metalloproteases produced by *Vibrio* species on Pacific oyster (*Crassostrea gigas*) larvae. Appl. Environ. Microbiol., submitted.

## Budget Justification

Salaries: The requested personnel budgetary items described in this proposal include sufficient support for the following:

Dima N. Gharaibeh, M.S., Research Technician @ 100% effort (1.0 FTE at rate of \$36,000). Dima will be involved in all aspects of this work. She has had previous training in general microbiology and molecular biology techniques. Importantly, she has previously worked at MitoSciences on the development and optimization of lateral flow dipsticks. She has been in the PI's lab for almost a year and is a diligent worker well suited for the proposed work. She is currently involved in developing a quantitative PCR (qPCR) assay for the detection of *V. tubiashii* bacteria.

Fringe Benefits: Benefits are 63% of the technician's salary (as per institutional guidelines).

Material and Supplies: Materials and Supplies include standard laboratory and microbiology reagents (including plastic ware, bacterial growth media, His-tagged protein purification kits, etc.) at \$4,883. Costs for reagents/materials for the development of the dipstick assay (which includes gold colloid, nitrocellulose membranes, etc.) are estimated at \$4,795. The total cost for all materials and supplies will be \$9,678.

Equipment. Funds are requested to purchase a Hamamatsu ICA-1000 dipstick reader (\$5775; <http://www.mitosciences.com/ica1000.html>) in year 2. The Hamamatsu ICA-1000 is a PC-based system specifically designed to read lateral flow devices that employ conjugated gold and provides a complete software system for measuring and analyzing lateral flow dipsticks.

Other Direct Costs: Funds are requested for the custom monoclonal antibody production for the first year. Abbomax, Inc. (San Diego, CA) will provide up to 12 ELISA-positive provided as pre-clones that can then be screened for their suitability in the proposed ELISA and dipstick assays for \$5,500 ([www.abbomax.com](http://www.abbomax.com); Cat# 60003).

**APPENDIX 4**

	Requested funds	Matching funds
A. TOTAL SALARIES AND WAGES	\$36,000.00	
B. TAXES AND BENEFITS	\$22,680.00	
C. TOTAL PERMANENT EQUIPMENT	\$5,775.00	
D. TOTAL EXPENDABLE SUPPLIES AND EQUIPMENT	\$9,678.00	
E. TOTAL OTHER COST	\$5,500.00	
F. GRAND TOTAL REQUESTED	\$79,633.00	