THREATS TO BIVALVE AQUACULTURE AND FISHERIES: THE INFLUENCE OF EMERGING DISEASES AND ENVIRONMENTAL CHANGE

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Project Summary

Shellfish are important species for our growing marine shellfish aquaculture industry and play critical roles in our marine ecosystems, an environment that is increasingly threatened by environmental change. In the Pacific Northwest the environment has changed in a manner that has contributed to increase mortality of bivalve larvae in hatcheries and also appears to have decreased natural recruitment. Several local shellfish hatcheries, upon which nearly the entire bivalve culture industry relies, have experienced severe losses (e.g. up to 59%) over the past two years. Several factors have been attributed to this problem including temperature, ocean acidification, and re-emerging pathogens. Given the large-scale environmental change observed in our marine ecosystems and the relationship of host stress response and pathogen virulence with environmental conditions, it is critical to examine the problems facing bivalve larvae from a regional perspective by systematically assessing how the environment influences the spread of disease and the ability of oysters to effectively respond to stress. The goal of this proposal is to characterize the factors that threaten the aquaculture industry and wild shellfish. The primary approaches include a series of laboratory experiments and environmental sampling. For the current proposal has been developed to test the following hypothesis: ***Environmental stressors (elevated temperature and reduced pH) will enhance disease expression (vibriosis and or OsHV) and reduce larval bivalve survival.*** More specifically we will test the impact of single and multiple biotic and abiotic stressors on larval bivalves with a focus on the most economically important regional species, the Pacific oyster (*Crassostrea gigas*). In order to assess the impact of biotic and abiotic environmental factors on bivalve health, we will also assess the abundance of oysters and other larvae in Willapa, Dabob, and Netarts Bays in relation to water quality parameters (pH, temperature, dissolved oxygen, salinity, alkalinity, chlorophyll A, and pathogen load). The specific research objectives are to: ***1) Characterize the interrelationship of altered environmental conditions, pathogen, and oyster response under controlled conditions and 2) Identify factors in Pacific Northwest hatcheries and in the wild that are associated with poor oyster larvae survival.*** Upon completion of this research we will have a better understanding of how environmental change will impact our marine ecosystem. This information will allow us to better predict mortality events, improve hatchery practices, manage wild populations, and develop improved broodstock. Furthermore, marine bivalves are an excellent sentinel species for environmental perturbation and the novel bio-monitoring procedures developed in this project could easily be transferred to other species and systems.

1. Project goals and objectives

1.a Introduction

Need for the project:

This proposal meets several specific priorities listed under the SK solicitation document, section Aquaculture: "**marine aquaculture including hatchery technology**, ……**disease diagnostics and control**….". We aim to improve hatchery production through collaborative experiments, and to develop diagnostic methods for specific pathogens and stressed bivalves. Collectively these tools will enable shellfish aquaculturists to better control threats to their production.

The bivalve culture industry plays an important economic role in coastal communities on a global scale, particularly in the Pacific Northwest of the United States. This growing marine aquaculture industry is becoming more reliant on hatchery-produced larvae and seed as opposed to more historical methods of capturing wild sets of larvae for seed (juvenile) production. In Washington state, shellfish growers in Willapa Bay still rely on the capture of natural sets of Pacific and Olympia oyster larvae. However the Willapa Bay industry, like the rest of the shellfish farmers in the State, has begun to benefit from hatchery technology as a more controlled and reliable source of larvae and seed for production. Recent losses in both hatchery (see letters) and wild sets (e.g. in Willapa Bay, Brian Sheldon, Northern Oyster Company, personal communication) have had, in some cases, catastrophic impacts on the few hatcheries supplying seed throughout the western region. Many farms rely on the purchase of larvae and seed produced elsewhere (e.g. no commercial bivalve hatcheries exist in California) for grow-out at their farms. Washington state currently has three bivalve hatcheries in commercial production, and a fourth exists in Oregon. Two of these facilities have been severely impacted by water quality (low pH and elevated temperatures) and disease issues (e.g. caused by *Vibrio tubiashii*) that have resulted in up to 59% losses over the past two years (Elston et al. 2008, Joth Davis, Taylor Resources, see letter of support. Sue Cudd of Whiskey Creek Oyster Hatchery estimates up to 80% losses at their facility (see letter of support). Seed losses associated with pathogens have also impacted young seed oysters in parts of California due to an ostreid herpes virus (OsHV, Burge et al. 2006, 2007) alone or in combination with *V. tubiashii* infections (Colleen Burge, personal observation). At the most recent (October 2008) Pacific Coast Shellfish Growers Association meeting in conjunction with the Pacific Coast Section of the National Shellfisheries Association, where aquaculturists and scientists met and exchanged information, three sessions were devoted to these problems, including the entire plenary session. It is imperative that the impacts of these stressors, both abiotic and biotic, on larval bivalve survival are investigated.

We have developed a multidisciplinary and integrated approach to understand the impact of single and multiple stressors on the larvae. We propose to develop diagnostic tools to enable more rapid and sensitive diagnosis of the pathogens. We will also develop tools to identify how bivalves respond to various stressors. Upon completion of the proposed project we will provide the shellfish aquaculture industry with powerful tools to identify, manage, and ultimately overcome these severe impediments to the growth and success of this important marine aquaculture industry.

1.b Background

Shellfish Our oceans are changing, thereby influencing the biology of many organisms, including those with calcareous skeletons such as shellfish. Shellfish are a critical component of our marine ecosystems, an environment that is increasingly threatened by environmental change. As benthic filter-feeders, oysters play an important role in estuarine food webs and contribute to the removal of excess organics, nutrients, and particulates (Newell et al. 1999; Rice 1999; Rice et al. 1999, Officer et al. 1982). Nutrients such as nitrogen and phosphorus that are not incorporated into oyster tissue are excreted and can then be utilized by keystone plants such as eelgrass (Newell 2004). Plants also benefit from increased sunlight associated with the oyster's ability to improve water quality via particulate removal (Newell et al 2002). Oysters commonly grow, and are farmed, in aggregations that have been shown to enhance biodiversity (Ferraro and Cole 2007). Such structures are integral not only for larval oysters, but for other organisms such as worms, snails, crabs, fish and birds that utilize the structures as habitat. Shellfish such as oysters are also carbon fixers, incorporating carbon into their shell thereby helping to reduce carbon dioxide levels (Newell 2004). Clearly, effective ecosystem function and success of our local shellfish aquaculture industry is predicated on the ability to produce healthy larvae. *In the Pacific Northwest the environment has changed in a manner that has contributed to increased mortality of bivalve larvae and decreased natural recruitment* (see text below and letters of support for details).

During 2008 there have been reports of no barnacle settlement in Netarts Bay, OR (Alan Barton, Whiskey Creek Oyster Hatchery, personal communication) and a lack of wild larval catch in Willapa Bay, WA (Brian Sheldon, Northern Oyster Company, personal communication). Given the recently measured summer concentrations of the shellfish pathogen *V. tubiashii* in Willapa Bay that are consistent with bivalve larval failures (Ralph Elston, unpublished data), it is possible that this pathogen alone or in combination with local abiotic parameters (e.g. temperature and pH) is impacting wild populations of marine invertebrates along the Pacific Coast. Poor recruitment has not been observed along the entire west coast of the US and not all hatcheries are faced with dramatic problems. However clearly conditions have changed with negative consequences for oysters. It is crucial that we scientifically examine the current situation to be better prepared for the future. Understanding stressors contributing to epidemics is crucial for management of bivalves in both natural populations and culture facilities.

Regardless of whether we are observing conditions directly related to large-scale climate processes, we certainly have the opportunity to study a situation that will provide valuable insight into how our fisheries will respond to changes related to temperature, acidification, and re-emerging pathogens. Completion of this project will not only provide a valuable knowledge base to protect shellfish in the region but our collaborative approach will serve as a model for studies for other fisheries. Furthermore, marine bivalves are an excellent sentinel species for environmental perturbation and the novel bio-monitoring procedures developed in this project could be transferred to other regions, increasing the understanding of how shellfish and finfish fisheries will be impacted in the future.

Bivalve Shellfish Aquaculture in the Pacific Northwest: Current status and threats Pacific oysters are a key cultured shellfish species in the US with production estimated at 40,601 tonnes (WRAC, 2004; FAO 2006). Washington State produces approximately 87% of US Pacific oysters and California produces most of the rest. In addition to harvest-sized product, 35 million Pacific oyster seed were produced in 1995 (WRAC 2004) and production has increased since that time. Oyster farming is estimated to contribute over $120 million annually to the region's economy (WRAC, 2004).

Several factors have been identified as contributing to the observed decline in hatchery and wild oyster populations. However we do not have a clear understanding of how these environmental stressors function at either the organismal or the ecosystem level. The research proposed here focuses on the Pacific oyster (*Crassostrea gigas*), an important natural resource in the Pacific Northwest with substantial economic and ecological importance. Integral to our understanding of the oyster is how environmental conditions influence oyster disease.

More specifically, in recent years bivalve hatcheries have been faced with a variety of problems with larval production that have occurred contemporaneously with reports of the absence of marine shellfish larvae at natural sites that were once very productive (Brian Sheldon, Northbay Oyster Company, personal communication). In some mortality events experienced in commercial hatcheries, specific, associated factors have been identified. Aside from typical problems with temperature control and facility control, specific and virulent shellfish pathogens have been identified. At one hatchery *Vibrio tubiashii* caused losses of 59% of Pacific oyster larvae produced (Elston et al. 2008, Sue Cudd, Whiskey Creek Oyster Hatchery: See letter of support). In cases where no infectious disease causing agent has be identified other environmental conditions appear to be correlated with mortality including pH shifts (Alan Barton, Whiskey Creek Oyster Hatchery, personal communication; Ed Jones, Taylor Resources Hatchery, personal communication). In the natural near shore environment, the pH of seawater may change (i.e. due to biological activity, H_2S production, etc); such pH shifts may play a role in the success of bivalve recruitment. Calabrese and Davis (1966) examined the pH tolerance of two species of bivalve larvae (*Crassostrea virginica*, the eastern oyster, and *Mercenaria mercenaria*, the hard clam) and found that larvae of both species were tolerant to a wide range of pHs at their one experimental temperature. However, it is important to note that their 'normal seawater' had a pH range of 7.4-7.7, which is lower than that typically encountered locally in the Pacific Northwest of the US. These authors also noted that developing embryos were much less tolerant to low pH than were larvae (Calabrese and Davis 1966). This observation supports recent experiences in which the fate of larvae seemed to depend on what they experienced during the initial 48 hr post-fertilization

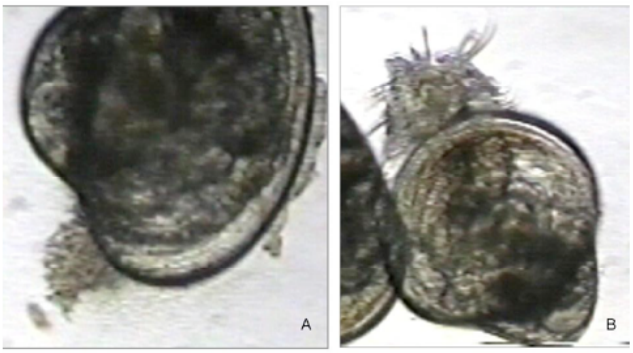

Figure 1. Pacific oyster larvae infected with *Vibrio tubiashii* isolated from a hatchery A. Swarming bacteria surrounding larva and its necrotic tissues. B. Infected larva with deformed velum. Figure modified from Estes et al 2004.

(Sue Cudd, Whiskey Creek Oyster Hatchery; personal observation). Thus, it is crucial to empirically assess the impact of specific environmental parameters on early life stages of species of local importance. Although low pH seawater has been associated with larval losses at two local hatcheries, the relationship between low pH and disease remains unclear. One key objective of this proposal is to better understand the impact of the changing marine environment of larval health, including synergistic effects with other stressors such as pathogens.

1.c Stressors facing shellfish larvae

Disease- Across the United States, losses of oysters associated with disease has increased over the past few decades with expansion of known disease agents (such as *Haplosporidium nelsoni* and *Perkinsus marinus*) and the introduction of emerging diseases such as those caused by the oyster herpes virus (OsHV) and *Vibrio tubiashii* (Figure 1). The impact of diseases on wild and cultured oysters is considered a leading impediment to the oyster industry within the US, clearly affecting the health of some of key estuaries such as the Chesapeake Bay (Andrews 1988, Burreson et al. 1996, Ragone Calvo et al. 2003). In addition to ecological impacts, diseases have resulted in economic losses thereby hampering the sustainability of oyster culture in several US areas including the mid-Atlantic, Gulf and West Coasts. Serious disease issues are emerging in some locations in Pacific Northwest of the US, which could also affect local estuarine ecosystem dynamics and marine-based economies.

Vibrio tubiashii

Locally, certain hatcheries along the west coast of the US have been impacted by vibriosis. This disease is caused by members of the genus *Vibrio*, including *V. tubiashii*, the most frequent and problematic pathogen over the past few years. Disease outbreaks have resulted in a lack of seed for many farms (John Finger, Hog Island Oyster Company, and Drew Alden, Tomales Bay Oyster Company; personal communication) and partial closure of at least one hatchery (Jonathan Davis, Taylor Resources, Inc., personal communication). In fact, *Vibrio* species are pathogenic to a wide variety of marine invertebrates. Although larvae are most commonly affected, adult shellfish may also be susceptible [e.g. mature abalone in France (Nichols et al. 2002) and manila clams with brown ring disease throughout Europe (Paillard et al. 2004)]. In 2006 and 2007 *V. tubiashii* re-emerged as a significant pathogen limiting survival and production of Pacific oysters (*Crassostrea gigas*), kumamoto oysters (*C. sikamea*) and geoduck clams (*Panopea abrupta*) with losses approaching 59% (Elston et al. 2008) and estimated to be as high as 80% (Sue Cudd, personal observation, see letter of support). Strains isolated over the past few years cause 100% mortality within 3-5 days at concentrations of only 100 bacteria/mL (Estes et al. 2004, Elston et al. 2008). For reference, natural blooms associated with mortality event were documented at 1.5 $x10^5$ /mL (Elston et al. 2008). In all of these cases, elevated seawater temperature was a key factor associated with the vibriosis epidemic (see below). ***Although vibriosis has plagued the bivalve shellfish industry for many years, recent recurrent epidemics have heightened our awareness of this problem and we now realize that the magnitude of these epidemics is larger than we previously realized***. Aside from causing losses in hatcheries vibriosis may severely impact larvae of a variety of natural marine species including more than previously suspected.

The precise factor(s) contributing to the re-emergence of *V. tubiashii* are likely complex however Elston et al. (2008) show a clear association with sea surface temperature. As shown in Figure 2, during July and August in 1998 and 2007 when sea surface temperatures were elevated in conjunction with periodic upwelling, significant blooms of *V. tubiashii* (section B on graph in Figure 2) occurred and significant hatchery losses of bivalve larvae were observed.

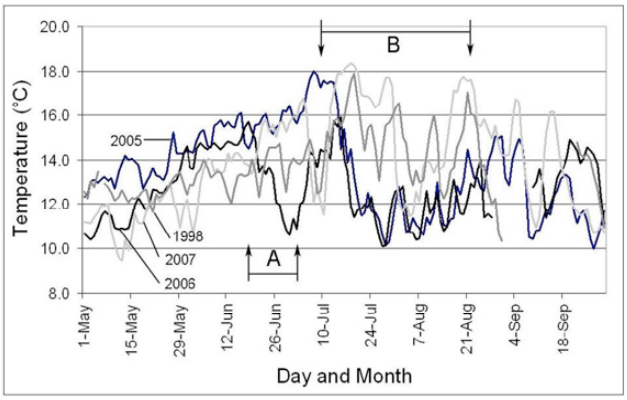

Figure 2. Surface seawater temperature, May-September 1998, 2005, 2006 & 2007, from the NOAA Stonewall Banks buoy located near Newport, Oregon. Bracket A denotes a period of rapidly falling temperature in 2006 coincident with a transient vibriosis outbreak at the Tillamook, Oregon hatchery. Bracket B shows the temperature profiles in 1998 & 2007 when production dramatically dropped at the hatchery coincident with mean high temperatures during this period; oscillations were caused by intermittent upwelling and intrusion of warmer-than-normal surface seawater. Figure from Elston et al. 2008

In addition to a relationship with temperature, pH may also influence pathogen survival and its ability to infect hosts. *V. tubiashii* appears to have greater viability at relatively low pH (Figure. 3). Local hatcheries have recently observed unusually low pHs of incoming seawater that has ranged from a low of 7.2 to 8.0, nearly optimal pHs for this bacterium based on our laboratory experiments. These preliminary results suggest that pH within the range of what is experienced in the region impacts *V. tubiashii* and provide partial basis for our hypothesis. Nothing is known regarding how this is occurring and what impact these physiological changes have on oysters.

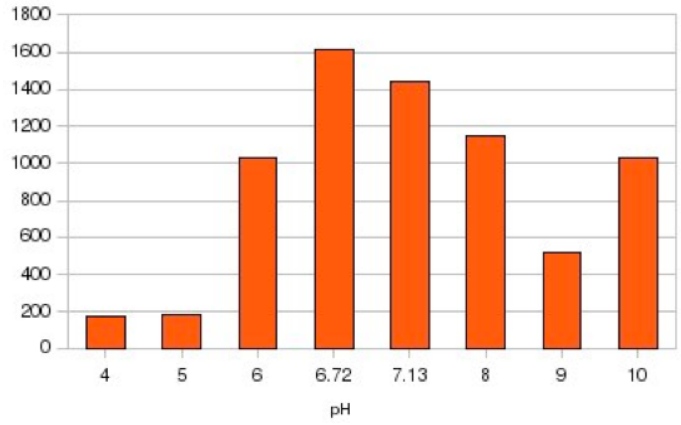

Figure 3. Relative growth of *V. tubiashii* in media adjusted to range of pHs (Roberts unpublished). Y-axis is arbitrary RNA quantity.

Based on the original characterization of vibriosis (Tubiash et al. 1965), Brown (1973) suspected the involvement of a toxin due to the rapid mortality of oyster larvae within 18 h of exposure. Two major toxins have been described in association with vibriosis, one degrades connective tissues and the second is heat stable and ciliostatic, resulting in cessation of swimming (Nottage et al. 1989). The expression of toxins, presence of certain outer membrane proteins, and pilis of some *Vibrio* species (e.g. *V. cholera*) have been shown to be regulated by changes in salinity, amino acid concentration, temperature and/or pH (Sperandio et al. 1995, Beaubrun et al. 2007). These data also support the hypothesis that environmental stressors will enhance disease expression.

While there is less known about virulence in *Vibrio tubiashii* compared to clinical (human pathogenic) *Vibrio* strains, recent research has identified several mechanisms. This bacterium is now known to produce multiple exotoxins, including a metalloprotease, a heat stable ciliostatic toxin, and a chitinase (Nottage et al. 1989, Hasegawa et al. 2008, Marushchak et al. 2008). Like other vibrios, *V. tubiashii* expresses a number of previously recognized outer memebrane proteins (OMPs or porins), which may function in bacterial attachment, transfer of small molecules and disease development in its host (Aeckersberg et al. 2001, Beaubrun et al. 2008). Recently our lab has identified chitinase as a potential virulence factor in *V. tubiashii* as gene expression is signficantly upregulated when *V. tubiashii* is in proximity to Pacific oysters as compared to controls (Marushchak et al. 2008). In a related line of research we also used proteomic approaches to identify proteins differentially expressed in *Vibrio tubiashii* when in proximity to oysters. By comparing differential "spot" patterns and protein characteristics (e.g. isoelectric point and molecular weight) with protein databases of a completely sequenced vibrio strain, *Vibrio paraheamolyticus*, we were able to putatively identify these proteins (Figure 4). As might be expected outer membrane proteins were identified (maltoporin and ompK). Interestingly, other putative identifications for these upregulated proteins include flagellins. Synthesis of flagella is associated with the physiological change vibrios undergo when transforming from a passive stage to an active more motile stage where the actively swim. In this case, flagellin expression indicates that vibrios "know" oysters are present and will thus engage in swarming behavior. Environmental conditions that have been shown to be required for flagella expression (and thus swarming behavior and perhaps accumulation in oysters) include iron-limiting conditions and viscosity (McCarter et al. 1988, McCarter and Silverman 1989). Taken together, our preliminary results provide excellent target genes to characterize expression in *Vibrio tubiashii*, which will add substantially to our understanding of how changes in environmental conditions influence pathogen virulence and general physiology

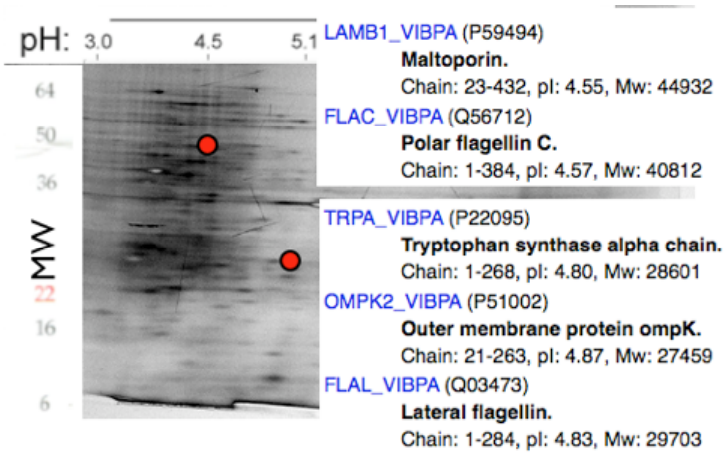

Figure 4. Two dimensional electrophoresis gel of proteins expressed by *Vibrio tubiashii* when exposed to oysters. Circles indicate proteins upregulated compared to control gel (not shown). Putative identification for each protein provided at right. Expression levels of genes encoding these proteins will be candidates to for Reverse Transcription PCR assays carried out to characterize physiological response of *Vibrio tubiashii* to changes in environmental conditions (i.e. temperature, pH, co-occurrence with *C. gigas*, co-occurrence with OsHV).

Ostreid herpes virus (OsHV)

Herpesvirus infections have been identified in oysters as early as 1972 in *Crassostrea virginica* in Maine (Farley et al. 1972), but were not identified again in the US until 2002 when it was detected in Tomales Bay, California infecting seed Pacific oysters (Friedman et al. 2005). The virus infecting oysters in Tomales Bay appears to be similar to herpes-like virus(es) that have caused losses of larval and seed oysters of several species in New Zealand (Hine et al. 1993) and France (Nicolas et al. 1992 and Renault et al. 1994 a,b) since the early 1990s. This virus has also been detected in multiple Asian countries, including Korea, China, and Japan (Moss et al. 2007) and Mexico (Vazquez-Juarez et al. 2006). In 2002, an oyster herpesvirus screening project sampled oysters from the east, Gulf, and west coasts of the US, employing both histopathological and molecular techniques. Results from this survey suggest that OsHV was only present in Drakes Estero and Tomales Bay, CA (Friedman et al. 2005, Strenge et al. in prep.); in 2006, however, OsHV was detected in a group of Pacific oysters in Totten Inlet, Washington State (Burge et al., unpublished data). Since 1993, catastrophic losses of juvenile Pacific oysters have occurred on a nearly annual basis in Tomales Bay, California where OsHV has been detected as early as 1995 (the earliest test date), and OsHV has been associated with these losses (Burge et al. 2006, 2007). Virus reactivation in individuals carrying a latent state virus is likely triggered by increased temperature as high temperatures have been associated with mortalities in Tomales Bay (Burge et al. 2006, 2007). ***The virus isolated from oysters collected in Tomales Bay has been demonstrated to cause 100% mortality in Pacific oyster larvae within 9 days*** (Burge and Friedman, unpublished data.; Figure 5). In Tomales Bay in 2008, both OsHV and suspected shellfish *Vibrio* species were detected during mortality events of oysters, indicating that seed oysters that originated from WA hatcheries impacted by vibriosis were likely previously infected by *Vibrio tubiashii* and may be more susceptible to OsHV. We suspect that larval oysters infected with *Vibrio tubiashii* are also likely simultaneously susceptible to OsHV and hatchery diagnostic data suggests that co-infection is a common observation (Elston 1999). Additionally, in 2008, production of oysters in France were severely hampered by the co-occurrence of OsHV and *Vibrio splendidus* in both seed and adult oysters, an unusual occurrence (T. Renault, IFREMER, personal communication).

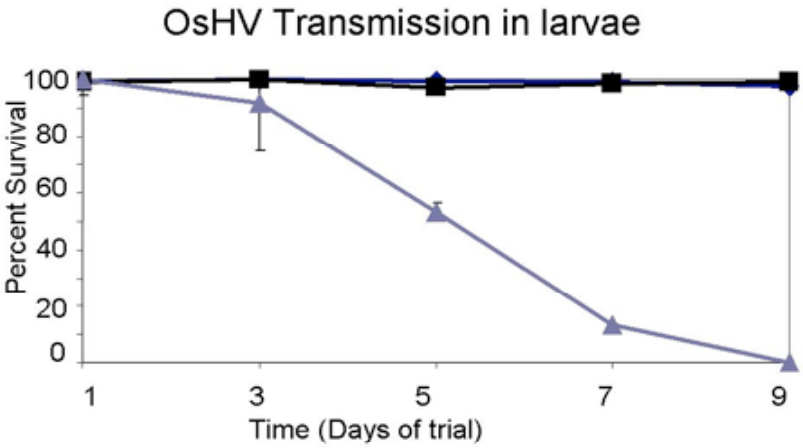

Figure 5. Experimental transmission of OsHV in Pacific oyster larvae.

Losses of oysters in the US to OsHV currently appear to be limited to young seed oysters in Tomales Bay, however, the ***recent detection of the virus in Washington state, the global existence of the virus, and the general lack of knowledge of requirements for OsHV reactivation and transmission implies there is potential for OsHV introduction into bivalve hatcheries.*** The lethality of this pathogen in conjunction with pH changes and current problems with *Vibrio tubiashii* would likely cause further production issues for bivalve hatcheries especially in light of current climatic changes experienced and predicted in the future.

Tools for pathogen quantification

In order to study pathogens as stressors to oysters it is necessary to be able to accurately quantify levels of the pathogens (eg *Vibrio tubiashii* and OsHV). For *Vibrio tubiashii*, current hatchery based diagnosis utilizes a combination of culture, antibody reactivity and confirmation by PCR methods for presence of metalloprotease and hemolysin genes (Ralph Elston et al.; personal communication). The PIs on the current proposal were recently awarded a seed research award for the University of Washington's School of Aquatic and Fishery Sciences to begin the development of a simplified and efficient quantitative molecular assay. We are currently taking several approaches to identifying appropriate targets. We have selected two sets of PCR primers based on the chitinase gene and intergenic spacer (see Section 6.c for details). The continued development of quantitative real-time PCR (qPCR) methods via this Saltonstall-Kennedy grant will enable high throughput and more rapid and specific, quantitative assessment of this bacterium in a wide range of sample types (e.g. bivalve or other animal, plant, sediment, seawater, etc). We will employ classic bacteriological and microscopic methods for confirmation of our molecular results as needed. We have developed a qPCR assay for OsHV (Burge et al. in manuscript) and will employ this assay for identification and quantification of the herpes virus in samples. This latter assay reliably detects 3 genome copies of OsHV, and is a cheaper and faster method of detecting virus gene copies than traditional microscopic methods (e.g. electron microscopy). As the OsHV is non-culturable, molecular methods are needed for quantification, particularly for life stages older than larvae as this virus does not readily form visual signs of infection as it does in larval bivalves (Friedman et al. 2005, Burge et al. 2006). Despite the presence of nuclear changes suggestive of herpes virus infection in larvae, molecular methods are needed for accurate quantification of viral gene copies (Batista et al. 2007). As needed to confirm active infection, we will employ electron microscopy, histology and *in situ* hybridization methods for confirmation of selected samples.

1.c Stressors facing shellfish larvae **continued**

Environmental Factors - In addition to biotic stressors, such as disease agents, abiotic environmental factors may act as stressors either individually or in synergy with other factors. It should be noted that the impact of disease and environmental conditions are integrally linked. As described by Snieszko (1974) disease results from a disruption in the equilibrium among the host, the pathogen and the environment that leads to the ability of the pathogen to infect, replicate and cause disease within the host. Frequently such shifts in the host-parasite relationship favoring disease are associated with climatic change such as ocean warming and potentially acidification (Jackson 2008).

As with most aquatic organisms, temperature stress (both chronic and acute) is a common insult that compromises a variety of physiological processes and cellular components. Temperature is directly correlated with metabolism and thus greatly influences growth. Chronic thermal stress has been demonstrated to impact the oyster immune response by reducing the killing index or ability of Pacific oyster hemocytes to kill bacteria (in this case of a pathogenic *Vibrio campbellii*) (Allen and Burnett 2008). In addition, an acute thermal stress (e.g. a shift from 20ºC to 28ºC for 2 days) has been demonstrated to reduce oyster immune function and was postulated to lead to increased disease susceptibility (Hagaret et al. 2004). These studies have been conducted on older animals from which hemolymph may be withdrawn. In addition to examining the impact of selected stressors on larval survival and growth, we will employ molecular methods to better understand their physiological response (including the immune response).

While water temperature has probably received the most attention in regard to its effect on oysters, pH has received little attention over the last decade. Given the implication of pH shifts associated with elevated atmospheric carbon dioxide, attention to this area of research is increasing. Shifts in CO_2 levels expected for the year 2100 (740 parts per million) were mathematically and experimentally found to significantly reduce the ability of calcareous organisms, including bivalves, to produce shells (Feely et al. 2004, Matsui et al. 2007). Friedman (Co-PI on current proposal) has observed a complete loss of larval gastropod shells upon exposure to a pH of 7.6 after 24-48h (unpublished data.). A similar low pH (~7.6) was found to be associated with a mortality event in a local hatchery (Ralph Elston; personal observation). It is unlikely that this pH directly killed the oysters, but the drop was symptomatic of, or enabled, some other biological process. Shellfish are influenced by pH and extreme acidic or basic conditions can be detrimental. While studying Eastern Oysters (*Crassostrea virginica*) on the Atlantic coast, Prytherch (1928) concluded that low pH inhibited spawning. Loosanoff and Tommers (1947) observed decreased filtration in adult oysters at pH below 7.0. In 1966, Calabrese and Davis carried out an elegant study examining the pH tolerance of eastern oyster larvae at a temperature of 25.1ºC (Calabrese and Davis, 1966). They found that the range for normal survival of oysters larvae was pH 6.25 - 8.75, the range for a normal rate of growth was 6.75-8.75, and the optimum pH was 8.25 - 8.50 (Calabrese and Davis, 1966). They concluded that reduced larval performance was attributable to the negative impact on ciliary movement. Even though the experiments carried out by Calabrese and Davis were completed over 40 years ago, the last sentence of the manuscript eerily predicted where we might be today "*It is apparent, therefore, that heavy siltation, or any pollution that can change the pH of tidal estuarine waters, could cause failure of recruitment of hard clams and oysters.*" It is critical to understand the influence of abiotic factors on Pacific species that reflect local conditions as proposed in our proposal.

It is likely that pH fluctuations in the Pacific Northwest were related to local biological processes and not directly related to increased global CO_2 levels. However this event does demonstrate the potential for changes in water quality to negatively affect biological processes. By studying pH shifts that are representative of current "real-world" changes, we will be able to better predict how such pH declines may affect natural and farmed populations in the future.

1.d Oyster biology and the stress response

As indicated, larval and early juvenile oysters are the most susceptible to insults facing hatcheries on the West Coast and elsewhere. Pacific oysters are broadcast spawners and once an egg has been fertilized it will develop into a ciliated trochophore larva within about 6 hours. A fully shelled veliger larva is formed within 12-24 hours of fertilization. The growth rate of larval oysters is temperature dependent (higher temperatures result in faster growth), but typically oysters will spend between 2 - 3 weeks as planktonic larvae before they develop a foot (known as the pediveliger larvae) and must find suitable substrate for settlement. The settlement stage of the life-cycle corresponds to intense anatomic and physiological changes as the larvae undergo metamorphosis into the juvenile stage known as spat.

While the development of oyster larvae has been fully characterized, a majority of research on the physiological stress response of oyster focuses on adult oysters. There are notable exceptions including Montagnami et al. (2005), Tirape et al. (2007), Hedgecock et al. (2007), Moran and Manahan (2003, 2004) and others. The following section will review the organismal responses of oysters in general to pathogens and environmental change. One primary component of the current proposal is to develop an assay to asses the oyster response.

Oysters and Pathogens- The immune system of an oyster is limited to an innate response comprised of a cellular and humoral components. Hemocytes are the primary defense cells, found in the hemolymph and interstitial spaces. Aside from playing roles in nutrition and cation transport, hemocytes are involved in inflammation, wound repair, oxidative burst activity and phagocytosis. Furthermore, hemocytes have the capabililty to secrete immune response factors including, antimicrobial peptides (Gueguen et al, 2006; Stensvåg et al, 2008), proteases and protease inhibitors (Zhu et al, 2006; Xue et al, 2006), lysozymes (Bachali et al, 2002; Matsumoto et al, 2006; Xue et al, 2007) and lectins (Kang et al, 2006; Kim et al, 2006; Gourdine et al, 2007). When shellfish are exposed to a pathogen (e.g., bacteria, virus, eukaryotic parasite) the response is often specific to the mode of action of the pathogen. Our lab has been involved in characterizing the immune response of the eastern oyster (*C. virginica*) exposed to the pathogen *Perkinsus marinus* (Roberts 2008). More recently, our lab has begun to characterize the immune response of *C. gigas* exposed to pathogenic *Vibrio* species, including *Vibrio tubiashii* (Roberts et al 2008a; 2008b, Marushchak et al. 2008). Figure 6 shows gene expression levels from oyster gill tissue taken from oysters exposed to *Vibrio* for 24 hours and controls. Given the significant regulation of these genes (Fig 6.) by the pathogen *Vibrio tubiashii*, these genes will be included in the gene expression assay developed to better understand stress response in oyster larvae.

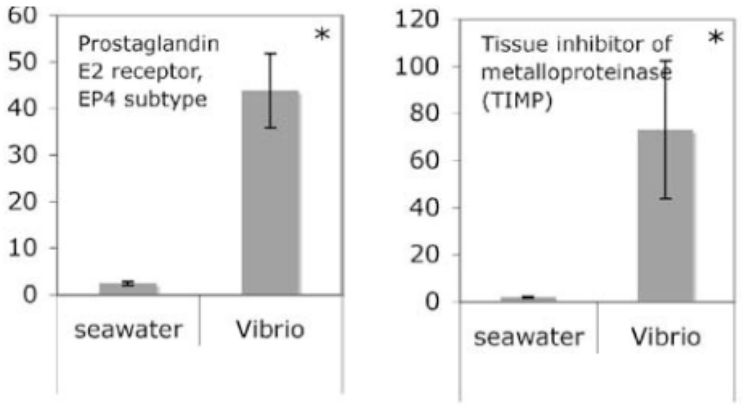

Figure 6. Differential gene expression of Pacific oysters in response to challenge with multiple *Vibrio* species. Similar patterns are observed when *V. tubiashii* alone is used alone. Left panel. Expression of gene similar to a prostaglandin receptor. Right panel. Expression of tissue inhibitor of metalloproeinase. Modified from Roberts et al. (2008b). Y-axis represents gene expression (fold increase over minimum). Asterisk denotes significant difference.

Oysters and Temperature - There has been substantial research on the impacts of environmental stress on shellfish in part due to the fact that shellfish are excellent bio-indicators. Temperature is a common stressor that is examined and is of particular interest given the predictions of large-scale climate variation. As noted by Harvell et al. (2002), a thermal change of only 1.5ºC can significantly alter the relationship between a potential pathogen and its host. One of the primary responses to temperature stress is expression of Heat Shock Proteins (HSPs). Heat-shock proteins are a family of molecular chaperones some of which are induced by heat and other stressors and others that are constitutively expressed. In both stressed and unstressed cells, these molecular chaperones are involved in folding, assembly, localization, secretion, regulation, degradation, and removal of other proteins (for review see Feder and Hofmann 1999). The need for these tasks is often higher when an organism is exposed to a stressor since this exposure commonly results in proteins assuming non-native (e.g., denatured) conformations. When routine cellular "housekeeping" processes are not carried out properly, there are usually negative effects on other physiological processes as the denatured proteins interact non-specifically with other molecules. Numerous studies have examined temperature induced HSP expression in invertebrates (e.g. Clegg et al 1998; Krebs and Feder, 1997; Singh and Lakhotia, 2000; Piano et al, 2002; Spees et al, 2002). Based on a recent study sequencing Expressed Sequence Tags in oysters we identified numerous HSPs from the Pacific oyster (Roberts et al 2008b).

Based on our work and by other research groups we know oysters respond to stressors including disease and temperature at the molecular level. In addition there are a number of studies that examine the transcriptomic response in relation to environmental estrogens, heavy metals, hypoxia, and sewage. Together these data provide the ground work for characterizing what is occurring to oysters in the Pacific Northwest and assist in monitoring oyster populations in the future.

1.e Research Objectives

Larval shellfish are always going to be vulnerable to lethal stressors, however engaging in a regional, integrative research effort to fully understand the underlying threats will benefit hatcheries across the country and also assist in managing wild populations. The long term goal of the project is to develop and implement a program to monitor and assess oyster larval health in hatcheries and the wild. For the current proposal we propose two specific research objectives to test the following hypothesis:

Environmental stressors (elevated temperature and reduced pH) will enhance disease expression (vibriosis and or OsHV) and reduce larval bivalve survival.

Specifically we will test the impact of single and multiple biotic and abiotic stressors on larval bivalves with a focus on the most economically important regional species, the Pacific oyster *Crassostrea gigas,* a commercially farmed species. In order to assess the impact of biotic and abiotic environmental factors on bivalve health, we will also assess the abundance of oyster and other larvae in Willapa, Dabob, and Netarts Bays in relation to water quality parameters (pH, temperature, dissolved oxygen, salinity, alkalinity, chlorophyl A, and pathogen load). The specific research objectives are to:

1) characterize the interrelationship of altered environmental conditions, pathogen, and oyster response under controlled conditions.

2) identify factors in Pacific Northwest hatcheries and in the wild that are associated with poor oyster larvae survival.

2. Project Impacts

This project has local, national and international impacts given that these problems are being experienced globally. Upon completion of this project we will have developed specific tools and have applied them to gain a better understanding of how factors working singularly and in unison impact oyster larvae. This information will be important for both improving hatchery conditions and managing natural populations. In order to fully understand the physiological impact of environmental conditions on oyster larvae we will develop, and field validate, a gene expression assay that will complement other observations designed to identify what factors are related to larval mortality. This assay along with the other sampling will lay the foundation for continued environmental monitoring in the region and across the country. The results of this project will provide valuable baseline data with which to compare future environmental monitoring data. The integrative approach to monitoring hatcheries and the wild using both traditional and molecular based methods will serve as a model for coordinated, integrated sampling throughout the United States. During the research project we will make the findings available to interested and the general public through a website hosted by the University of Washington similar to a site chronicling research on eastern oysters. [http://tinyurl.com/22nhbs]. Through our regional collaboration (two farms in WA, one in OR and one in CA), in conjunction with participation at the regional shellfish conference and national conferences, we will reach many members of this

industry as well as those in other areas nationally and internationally. This project will comprise the thesis of at least one graduate student who will publish his/her thesis. We anticipate at least three publications in peer-reviewed journals upon completion of this project.

3. Evaluation of Project

A primary milestone for the proposed project is characterizing how multiple factors ultimately impact larval shellfish survival. We have observed several instances of this anecdotally however there are limited studies that have been completed where this phenomenon was considered in developing a comprehensive laboratory and environmental sampling study. From an organismal perspective the identification of the oyster as well as *Vibrio tubiashii* response to stressors will be another successful accomplishment. For the activities of the current proposal related to sampling hatcheries and field sites, the data collection itself will be considered a success as this data will be valuable in evaluating future seasons and a contributing to a larger data set. This will include correlating hatchery conditions with adjacent local field sites. In other words vital baseline data will be collected that can be used for comparison in future seasons. We will submit a final report to Saltonstall-Kennedy and publish our findings in peer-reviewed journals such as Diseases of Aquatic Organisms, Global Change Biology, Aquaculture and Molecular Marine Biotechnology.

4. Need for government financial assistance

The proposed multi-state project is designed to understand impacts of stressors on shellfish larvae. While the stressors might vary in different regions, the promise of large scale climate variation will influence the appearance of new problems across the country. It is our intent that this type of integrated project will be a model for those interested in conserving our shellfish resources. In order to learn more about how pathogens and ecosystem conditions will change it is necessary to examine the interactions of multiple factors and field sample over a large geograhpic area. Therefore funds are requested from the federal government to support this project. We have also requested and received funding from the University of Washington's School of Aquatic and Fishery Sciences to identify genes indicative of changes in *Vibrio tubiashii* physiology and begin to develop molecular quantification of the pathogen. We have made significant progress on both fronts as highlighted in the Introduction with this seed money and are in need of the funds requested in this proposal to better understand and manage threats to bivalve aquaculture and fisheries. These data will contribute to the success of the project proposed here by providing essential tools to study not only *Vibrio tubiashii*, but OsHV and biotic stressors on bivalve health.

5. Federal, state and local government activities and permits

This project will interact with a Washington State Resource agency. We will obtain a Washington Department of Fish and Wildlife (WDFW) import permit for larvae from Oregon. The Friedman laboratory at UW is currently in possession of all needed import permits and renews its importation permits with WDFW annually.

6. Project statement of work

The overall goal of the proposed project is to further our understanding of how environmental factors influence shellfish hatchery mortality and local recruitment events in the wild. The two primary approaches will be to complete a series of laboratory studies as well as engage in a complementary environmental sampling in the Pacific Northwest. In the first part of this section we will describe the proposed research with detailed methodologies provided at the end of the section.

6.a Laboratory trials

Disease events are often linked to more than one stressor such as elevated temperature plus pathogen or elevated temperature plus low pH with or without a pathogen. In order to examine how specific environmental factors impact both pathogen and oysters a series of laboratory trials will be conducted. To begin with, only single variables will be manipulated, followed by dual and finally multiple stressor combinations. In our initial trials, the host or the parasite will be exposed to a range of levels of a specific environmental parameter (e.g. temperature and pH). We will then combine dual parameters to examine their influence on organismal survival and performance, followed by the application of multiple stressors (Figure 7). This iterative process will lead to a better understanding of physical and biological tolerances of bivalve larvae and selected pathogens.

All trials will be carried out in triplicate 500mL flasks to better reflect host-parasite relationships. The selection of this size container is based on previous work of Estes et al. (2004) who found volume size influenced the concentration of bacteria required to kill oysters. The flask experiments in the current proposal, though much small than hatchery scale production, have revealed lethal bacterial concentrations that were similar to that observed in natural epidemics (Estes et al. 2004, Elston et al. 2008).

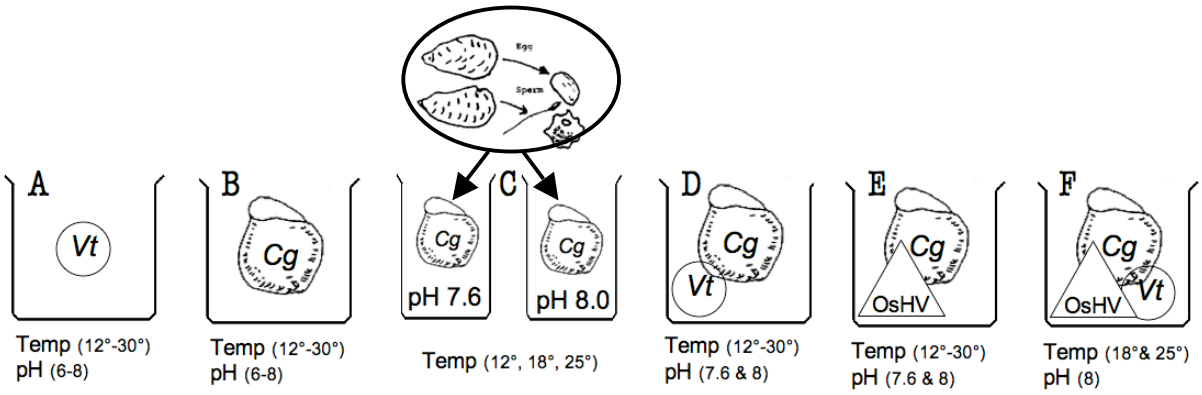

Figure 7. Schematic of laboratory trials to be carried out for the proposed project. testing the influence of stressor on the survival, growth and physiology of the pathogen and or the host as follows: A. Effect of pH and temperature on *V. tubiashii*. B. Effect of pH and temperature. C. Effect of exposing gametes and early larvae to varying pH and temperatures. D. Effect of pH, varying temperature and *V. tubiashii* (Vt). E. Effect of pH, varying temperature and the oyster herpes virus (OsHV). F. Effect of both pathogens at two temperatures on larval oysters. Note in D-F, physiology of both host and pathogen will be examined.

Trial A. *Vibrio tubiashi* (pH and temperature)
Vibrio tubiashii (Vt) will be grown in media with a pH range of 6-8. This will include 6 medias with pH's of 6.0, 6.8, 7.2, 7.4, 7.6, and 8.0. This range was selected based on reported variations in hatcheries, published reports and projections, and on our preliminary experiments already conducted (Figure 3). pH will be adjusted using carbonate, bicarbonate and HCl as needed. These *Vibrio tubiashii* growth trials will be carried out in 3^{o}C temperature increments from 12 ^{o}C to 30^{o}C. Samples will be taken at the following time points for all treatments: 0, 4, 8, 12, 24, 28, 32, 36, 48, 52, 56, 60 and 72 hours. To illustrate differential growth and physiology of *Vibrio tubiashii*, we will quantify bacterial replication and gene expression patterns for select genes as outlined in the specific method section below. Genes that will be targeted for measuring expression level differences e are described in section 1.b) and will include chitinase, metalloprotease, hemolysin, OMPs, and flagellins. (See specific methods section below for detailed description of methods). The reason both bacteria growth and gene expression analysis will be carried out is that while pathogen abundance and virulence are related in some instances (i.e. quorum sensing), abundance and virulence are the two primary factors that contribute to oyster mortality. Pearson's moment correlation (r) will be used to test for a linear relationship between gene expression, growth rate, survival and environmental condition (pH and temperature, singly and in combination). Differences in growth among treatments will be assessed by ANOVA.

Trial B *Crassostrea gigas larvae* (pH and temperature)
In order to evaluate the relative ability of larvae of different ages and levels of development, Pacific oyster larvae (e.g. D hinge, veliger and pediveliger) will be exposed to a variety of pH and temperature conditions as described above for *Vibrio tubiashii* (pH: 6.0, 6.8, 7.2, 7.4, 7.6, and 8.0; 3^{o}C temperature increments from 12 ^{o}C to 30^{o}C). Differences in survival will be assessed using the Kaplan-Meier method and log-rank test (S-Plus); differences in growth will be assessed using ANOVA. As above, Pearson's moment correlation (r) will be used to test for linear relationships between gene expression, growth rate, survival and environmental condition (pH and temperature, singly and in combination).

Trial C *Crassostrea gigas larvae* (pH and temperature)
Recent experiences at local shellfish hatcheries indicate that conditions larvae experience during the initial 48 hours greatly influences subsequent larval performance (Sue Cudd, Whiskey Creek Oyster Hatchery, personal communication). We will compare larval survival when animals are spawned into seawater within normal pH ranges as a control (e.g. 8.0) with those spawned into suboptimal, acidic conditions (pH 7.6). Currently, incoming seawater at three of the principle west coast bivalve hatcheries have ranged between 7.2 and 7.6 (Ed Jones, Taylor Resources, Colleen Burge, University of Washington, and Sue Cudd, Whiskey Creek Oyster Hatchery, personal communication). Hatchery personnel have noted that larvae that experience suboptimal conditions low pH (± pathogens) during the initial ~48 hr after fertilization performed poorly compared to those experiencing normal conditions.

To examine differential survival of larvae stressed early in their life history relative to those stressed later in the larval period, we will strip spawn male and female oysters, and gametes from each oyster will be released into two separate containers at the two selected pHs (7.6 and 8.0) to be tested. Treatment pH will be maintained as zygotes are rinsed and reared in 50L

containers. pH will be tested and adjusted every 12 hr, and seawater will be changed after 24 hr to separate hatched from unhatched larvae and will be changed daily thereafter. At 48 hr post-fertilization, we will conduct a crossover experiment in which half of the larvae at each pH will be split into two groups, one at the control pH of 8.0 and the second group at the test pH (e.g. 7.6). Larvae will be maintained at selected pHs for the remainder of the larvae period (17-24d). Trials will be conducted at 12°C (to reflect summer upwelling conditions), 18°C (to reflect summer maxima) and 25°C (to reflect maximal hatchery larval temperatures employed).

Mortality rates will be determined and any changes in behavior noted. As described below, DDPCR (Differential Display PCR) and qPCR will be carried out on samples to characterize changes in gene expression. Recall that ***a primary component of this project is to develop a gene expression assay that can be used in the future to help elucidate what stressors are present and how these influence normal oyster physiology***. Differences in survival will be assessed using the Kaplan-Meier method and log-rank test (S-Plus); differences in growth will be assessed using ANOVA. As above, Pearson's moment correlation (r) will be used to test for linear relationships between gene expression, growth rate, survival and environmental condition (pH and temperature, singly and in combination).

Trial D. *Crassostrea gigas larvae* (pH, temperature and *Vibrio tubiashii*)
In order to identify the combined effects of multiple stressors, oyster larvae will be cultured under conditions where pH, *Vibrio tubiashii*, and temperature are manipulated. The same range of temperatures described for the experiments above will be used, 12-30C. Two pH's will be used; 8.0 (normal) and 7.6. The quantity of *Vibrio tubiashii* in which larvae will be immersed for 24 hrs will correspond to the LD_{50} as determined by prior experiments. Thus, we will expose larvae with $3x10^4$ to $3x10^{05}$ cfu/mL in 500 mL flasks. Concentrations will reflect differences in lethal doses previously observed using the methods employed here (see Estes et al. 2004, Elston et al. 2008). Samples will be taken at 24 and 96 hours. Water will be changed daily (or needed) to maintain pH. Larval survival will be assessed and larvae samples archived at -80 for later gene expression analysis. *Vibrio tubiashii* samples will also be archived by taking 5ml of water, centrifuging and freezing pellet at -80. Differences in survival will be examined as outlined above for pH and the Pearson Moment Correlation (r) will be used to test for a linear relationship between survival and pH-temperature combination.

Trial E *Crassostrea gigas larvae* (pH, temperature and OsHV)
In this set of trials, we will expose oyster larvae to OsHV instead of *Vibrio tubiashii*. The same range of temperatures described for the experiments above will be used, 12-30C. Two pH's will be used; 8.0 (normal) and 7.6. Transmission of OsHV in the laboratory has been most successful (Figure 5) by using 250 mL flasks containing 100 mL of 0.22 um filtered seawater with 150 Units Penicillin and 150 ug Streptomycin ml^{-1}. Pen/Strep resistant *Vibrio tubiashii* have been selected in our lab. The amount of OsHV that will be used will include doses previously determined in our lab. Larval growth and survival will be assessed as above and larvae samples archived at -80 ^oC for later gene expression analysis.

Trial F *Crassostrea gigas larvae* (temperature, *Vibrio tubiashii* and OsHV)
Following completion of the trials described above, a multiple pathogen experiment will be carried out (Figure 7f) to elucidate the interactive effect of these two pathogens on oyster larvae as it is likely that the range of these two pathogens will continue to overlap as sea surface temperatures rise. As it is beyond the scope of the current proposal to examine this interaction at the full range of the different conditions described above (6 pHs, 7 temperatures, and 13 time points), we will begin to characterize this interaction at two temperatures (18 ^{o}C and 15 ^{o}C); pH will not be altered. Larval growth and survival will be assessed as described above and larvae samples archived at -80 for later gene expression analysis. Pathogen samples will also be archived by taking 5ml of water, centrifuging and freezing pellet at -80^{o}C. Host response and changes in *Vibrio tubiashii* physiology will be assessed through gene expression analysis (described below).

Crassostrea gigas gene expression assay development - An oyster gene expression assay will be developed that will be designed to 1) obtain information on how environmental stress impacts larval oysters, 2) be used as a common metric for monitoring oyster and ecosystem health. The assay will be developed in conjunction with the series of experiments described above. Upon completion this assay will include 12 genes and use quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) and/or Differential Display Polymerase Chain Reaction (DDPCR) technology as described below. For example, we might select DDPCR to identify which oyster genes are regulated when only pH is altered. We expect to obtain at least 3 target genes to include with targeted genes selected from our prior research and published information (Table 1). Our assay will incorporate specific stressors examined in this proposal (i.e. pathogen and temperature); however the assay will also be designed with other environmental stressors in mind. For example, as described in the introduction, pH can impact shell formation in larval shellfish. Several targets that are known to be involved in shell formation (i.e. BMP-R2, Nacrein) will be included in this assay. Other processes examined will include genes regulated by hypoxia, environmental estrogens, hydrocarbons, and heavy metals. Details of the molecular techniques and accompanying partial list of targets are below section 6.c.

6. b Environmental Sampling
The second primary component of the project involves sampling commercial shellfish hatcheries and natural sites in proximity to the hatcheries. This approach will allow us to directly study the stressors in the hatcheries, correlate conditions with wild sets, quantify pathogen levels in the environment, and field test the oyster gene expression assay.*Hatchery sampling* - Two commercial hatcheries will be involved in the proposed research. One is located in Netarts, Oregon and was the hatchery most impacted by the *Vibrio tubiashii* outbreak in 2007. The second commercial hatchery is located near Quilcene, Washington and has not experienced as significant mortality issues as the other hatchery. Sampling will occur at both hatcheries during the early spring and summer over two years. Water samples will be collected when the hatcheries begin production (around February) through August; the frequency of sample collection and analysis will vary with the parameter being tested. Water samples will be analyzed for temperature, pH, DO and salinity daily (or at least 4x per week); chlorophyll a, alkalinity, dissolved POM, and calcium will be assessed semi-monthly from February through May and weekly thereafter. Using molecular diagnostics water samples will also be analyzed for the presence of *Vibrio tubiashii* and OsHV. During each season, two oyster spawning events

will be sampled. This will include archiving four oyster larvae samples for each spawn that correspond to specific stages of development (i.e. trochophore, D-hinge veliger, mid stage veliger and pediveliger) for later gene expression anlaysis. This will be done by preserving the samples in RNAlater. Characteristics of each spawning event will be recorded including percent fertilization, and proportion surviving settlement and metamorphosis. In addition samples will be evaluated at the AquaTechnics laboratory for health diagnostics and bacteriological analysis including *Vibrio tubiashii* quantification, total *Vibrio* spp. enumeration and total culturable bacteria to compare with historical data, and for evaluation of OsHV presence. Additional water samples will also be taken that precisely correspond to oyster sampling and will be analyzed immediately for temperature, pH, DO, and salinity; samples with be transported to the University of Washington for determination of chlorophyll a levels, calcium and alkalinity. Physiological status (e.g. virulence) in *Vibrio tubiashii* will be assesed by carrying out reverse transcription PCR on select genes identified as part of research objective 1.

Figure 8. Map of study area showing two shellfish hatcheries (building icons) and three field sites (triangles).

Wild sampling - Environmental samples will be taken from three field sites in the Pacific Northwest. Two will correspond in location to the two commercial hatcheries, Netarts Bay and Dabob Bay. A third site, Willapa Bay, WA, will also be sampled. Willapa Bay was selected because of the dramatic reduction of bivalve larvae seen in the water column (Brian Sheldon, Northern Oyster Company, personal communication). In fact, the past summer almost no larvae were observed in plankton tows conducted in Willapa Bay (Brian Sheldon, Northern Oyster Company, personal communication). Sampling these field sites will be taken contemporaneously with hatchery samples during years 1 and 2. At each location plankton tows will be conducted weekly during the expected spawning time. From each tow, water, zooplankton and phytoplankton will be preserved separately. We will sort samples for bivalve larval enumeration and testing. Water samples will be analyzed for temperature, DO, chlorophyll a, salinity, alkalinity, dPOM, total bacteria, *Vibrio tubiashii*, total vibrio, and OsHV. Zooplankton samples will be processed and analyzed for the the presence of *Vibrio tubiashii*, total vibrio, OsHV and total bacteria. For bivalve larvae collected, one half of the sub-sample will be preserved in RNA later for gene expression analysis and the other half in 70% ethanol. Larvae will be identified via microscopy and PCR techniques (See Vadopalas et al. 2006, Wight et al. 2008). Briefly, our goal is to assess bivalve larval

presence but it would be helpful to examine the presence of several classes of larvae. Larvae will be quantified in large taxonomic groups as fish, crustacean, and molluscs (gastropod and bivalve). Native Olympia oysters and Pacific oyster larvae will be quantified by qPCR using the methods of Wight et al. (2008) and conventional PCR using the methods of Patil et al. (2005) that we will adapt to a qPCR platform), respectively.

6.c Specific Methodologies

Classic Microbiology - We will enumerate total heterotrophic bacteria, total vibrios, and *Vibrio tubiashii* according to the methods of Elston et al. (2008). Briefly, total heterotrophic bacteria will be enumerated by conducting standard plate counts using Marine Agar (Difco) and optical density (OD) after 1:10 serial dilutions in sterile seawater. The absorbance OD will be read in a Genios microplate reader (Tecan) at 490 nm. The OD will be regressed against a standard curve based on plate counts. Total vibrios will be enumerated by comparing plate counts on marine agar to that on TCBS plates supplemented with 1% sea salts. TCBS is generally selective for members of the family vibrionaceae. *Vibrio tubiashii* will be enumerated by assessing the proportion of sucrose positive (yellow as opposed to green) colonies grown on TCBS that test positive in both a serum agglutination test and an azocasein protease assay as described by Elston et al. (2008).

For selected water samples (see experimental sections above) we will enumerate *Vibrio tubiashii* associated with large zooplankton and flocculent material, zooplankton, phytoplankton and small suspended material, and seawater. To achieve this, duplicate 1L aliquots of seawater from each location will be sequentially filtered through a graded series of filters: 2 mm, 60 um, and 5um, respectively. The 'seawater sample' will be that which passes through the 5 um filters. If needed, additional levels of filtration will be added.

Molecular Microbiology - *Vibrio tubiashii* growth will be quantified via spectrophotometry versus standard curves from plate counts and qPCR using the reaction conditions and primers of Lee et al. (2002) who developed PCR primers to detect and distinguish a suite of *Vibrio* species from mixed samples (see Figure 9). As described in the Introduction we have also developed an assay that targets the chitinase genes and this qPCR may also be used for identification and quantification purposes. Prior to funding of the current proposal we will have tested both sets of primers on a suite of samples. The reason we are testing multiple targets is due to the unknown amount of sequence variation in various strains of *Vibrio* including within the multiple strains identified as *Vibrio tubiashii*. Ralph Elston, a Co-PI on the current proposal maintains a large number of these pathogens that we are in the processes of analyzing. Prior to the start of the current proposal a single assay will be validated and selected.

Briefly, DNA will be extracted from individual samples using the QIAamp® DNA Mini Kit (Qiagen) or Qiagen DNA stool kit, according to the manufacturer's protocol. The latter kit reduces the presence of PCR inhibitors from extracted nucleic acid samples, which can be a problem when isolating DNA from plankton. To further reduce the presence of PCR inhibitors, BSA will be added to each reaction (See Vadopalas et al. 2006, OIE 2007 for chapter on infection by *Xenohaliotis californiensis* that was written by Co-PI Friedman). Extracted DNA will be stored at -80C until use. Reactions will be conducted in 96 well microtiter plates in 25 µl volumes, containing 100mM Tris-HCl (pH 8.3), 16 mM $(NH_4)_2SO_4$, 3.5 µM $MgCl_2$, 0.01%

Tween 20, 0.04 U heat-labile UNG (Roche), 200 μM dATP, dCTP, dGTP, 140 μM dTTP, and 60 μM dUTP, 0.14x Sybr Green, 0.32 μM each primer, and 0.5 U Immolase™ DNA polymerase (Bioline). Real time reactions will be carried out in a MX3000 real time instrument (Stratagene), as follows: 20 ° C (10 min); 95 °C (7 min); 40 cycles 95 °C (30 s), 55 °C (1 min) and 72 °C (30 s) with a fluorescence read during the final 11 sec of each extension step. The reaction will be followed by melting curve analysis to confirm amplification of a single target.

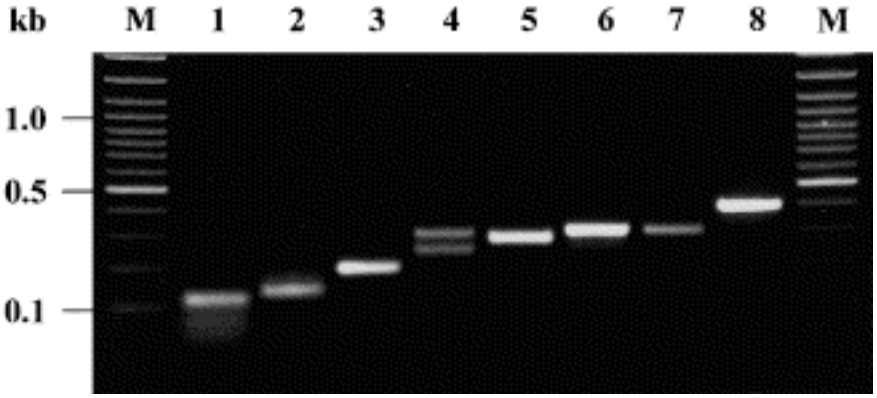

Figure 9. Agarose gel separating amplified DNA fragments from the intergenic spacer (IGS) region of several species of *Vibrio* using species-specific IGS primers. "Lane M – 100-bp DNA ladder (size marker); lane 1 – VNIG amplicon of *V. nigripulchritudo* ATCC27043; lane 2 – VPRO amplicon of *V. proteolyticus* ATCC15338; lane 3 – VCOS amplicon of *V. costicola* ATCC33508; lane 4 – VSPN amplicon of *V. splendidus* ATCC33125; lane5 – VSAM amplicon of *V. salmonicida* ATCC43839; lane 6 – VFLU amplicon of *V. fluvialis* ATCC33809; lane 7 – VDIA of *V. diazotrophicus* ATCC33466; and lane 8 – VTUB amplicon of *V. tubiashii* ATCC19105" Figure and legend from *Lee et al. (2002)*.

OsHV We will use the conventional PCR methods of Friedman et al. (2005) to identify infected groups and enumerate OsHV copy numbers according to Burge et al. (in preparation).
Conventional PCR: Briefly, we will employ the A3/A4, A5/A6 nested PCR primers of Renault et al (2000a). Briefly, each 20 μl reaction will contain 10x PCR buffer (10mM Tris, pH 8.3; 50mM KCl), 2.5 mM MgCl2, 8 μg BSA, 0.2 mM dNTP mix, 4 pmol each primer, 1 U *Taq* Polymerase (Promega) and 0.4 μl of template DNA. Following amplification with primers A3/A4, 0.4 μl of the reaction mixture will be added to the A5/A6 reaction mixture for the nested reaction. Duplicate nucleic acid amplifications will be performed with an initial denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final elongation at 72°C for 5 min. Products will be separated on 1.0% agarose gels containing 0.1 μg/ml ethidium bromide and visualized using a UV transilluminator.

Real-time PCR: An OsHV specific real-time quantitative PCR (qPCR) test has been developed in the Friedman Laboratory. Briefly, PCR primers designed to target sequence in the A fragment of the OsHV genome will be used to quantify viral load in oyster tissues against a standard curve. QPCR reactions (25uL) will contain the following: 1X SensiMix dU (Bioline), 300 nM primer, 1X SYBR Green Q solution (Bioline), 1X Uracil DNA Glycosylase, 8 ug of BSA, and template DNA. Thermal cycle conditions are as follows: 37°C for 10 min, 60°C for 2 min, and 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min, and 72°C for 30

seconds. As a control for DNA quality, all qPCR reactions without amplifiable OsHV DNA will be examined using the universal small subunit rDNA PCR test routinely used in our laboratory (Friedman et al. 2005). All reactions will include two positive control (plasmids) and three negative control reactions (water in lieu of template). All reactions will be carried out in 96 well microtitre plates in 25µl volumes.

Crassostrea gigas gene expression assay development - An oyster gene expression assay will be developed based on genes identified using DDPCR (see section 6.c) and RT-PCR on targeted genes selected from our prior research and published information. Table 1 is a partial list of genes that are differentially regulated in response to one or more environmental stressor. PCR primers will be developed using the software Geneious and Primer3. All oyster larvae samples will be processed and assayed as described in the specific methods section below

Table 1. Partial list of targeted genes - asterisk indicates gene confirmed to be expressed in oyster larvae.

Gene	Accession #	Stressor	Referenece
delta 9 desaturase	CB617498	hypoxia	David et al. (2005), Langdon (2008)
PG E2 Receptor	EW777722	Vt, Vibrio	Roberts et al. (2008)
CtMT-IV	AJ243263	heavy metals	Jenny et al. (2006)
AST	AJ496218	hydrocarbons	Boutet et al. (2006)
TIMP*	AF321279	bacteria, shell damage	Tirapé et al (2007), Montagnami et al. (2005)
BMP-R2	CU683618	shell formation	Miyashita et al (2008)
elongation factor	AB122066	normalizing gene	several
Cu/Zn superoxide dismutase*	ABF14366	metallic;organic compounds	Manduzio et al. (2004)
glutathione peroxidase*	ACB42236	heat shock	Tirapé et al. (2007)
MyD88*	DQ530619	bacteria	Tirapé et al. (2007)
LBP/BPI*	AY165040	bacteria	Tirapé et al. (2007)
Oxidative stress protein	EW778471	oxidative	Roberts et al. (2008)
Interleukin 17	EW779217	bacteria	Roberts et al. (2008)
estrogen receptor	AB259818	environmental estrogens,	Canesi et al. (2008)
Cytochrome P450 356A1	EF645271	sewage	Medeiros et al. (2008)
Multi drug resistance protein	EU073425	sewage	Medeiros et al. (2008)
Nacrein	CU683618	shell formation	n/a
flavin-containing monooxygenase 2	AJ585074	hydrocarbons, pesticides	Boutet et al. (2004)

Reverse transcriptase qPCR (RT qPCR). RTqPCR will be carried out on selected samples to better understand the physiological effects of selected stressors on oyster and *Vibrio tubiashii* biology. For both oyster and *Vibrio tubiashii* samples preserved as part of our trials and filed samples, RNA will be isolated using Tri-Reagent (MRC) and reversed transcribed with the QuantiTect Reverse Transcription Kit (Qiagen), which also eliminates genomic DNA carry-over. Quantitative, real-time PCR reactions (25uL) will contain the following: 0.5uL cDNA, 0.1uM forward/reverse primers, 1X Immomix Master Mix (Bioline) and 2uM SYTO13 (Invitrogen). An Opticon 2 thermocycler (Bio-Rad) will be used to quantify gene expression. Raw data will be analyzed using Real-Time PCR Miner Software (Zhao & Fernald, 2005).

Differential Display PCR (DDPCR). The Genefishing Differentally Expresssed Gene System (See-Gene) will be used which employs Annealing Control Primers (ACP) technology. The reason this approach is being used is that it is economical, fast, and easily enables comparison of a large number of different samples. The principle of ACP technology is based on the tripartite structure of a specific oligonucleotide primer (ACP) having 3'- and 5'- end distinct portions separated by a regulator and the interaction of each portion during two-stage PCR. The resulting PCR products will be separated on an agarose gel, and differentially expressed bands removed and stored for DNA sequencing. For the current proposal oyster samples will be compared under different conditions selected based on documented changes in behavior and/or mortality. For instance if under the same pathogen exposure yet different pH regimes oyster have significantly decreased performance, those samples will be compared to identify genes differentially regulated. This specific example would thus provide insight into how pH might compromise the immune system and provide an indicator of such compromise in field samples. PCR products will be cloned into TOPO TA pCR 2.1 (Invitrogen) and colonies of transformed bacteria will be grown for extraction of plasmid DNA. Templates will be prepared in a Rev Prep Orbit (GeneMachines) and the resulting cDNAs sequenced using a modified dideoxy chain termination method with Big Dye Terminator (Applied Biosystems). Sequencing reactions will be precipitated and pellets resuspended in Hi-Di Formamide with EDTA (Applied Biosystems) and analyzed using a 3730 Sequencer (Applied Biosystems). All sequences will be analyzed with NCBI Blast programs for similarity to known genes (Altschul, 1997). ClustalW (MacVector 7.2) analysis will be used for sequence pair-wise and multiple protein alignments. Previous use of this technique by the PI indicates one differentially expressed gene will be identified for ~10 primer sets used. As indicated previously we expect to ultimately identify at least 30% of the targets for the ~12 gene oyster expression assay using this method with the other targets similar to those listed in Table 1.

Water Quality. All parameters will be analyzed in triplicate. We will monitor temperature, pH, dissolved oxygen (DO) salinity, chlorophyll a (Chl a), calcium, alkalinity and suspended particulate organic matter (POM). Temperature will be continuously monitored with a HOBO TidbiT temperature datalogger set to collect thermal data hourly. Temperature will also be monitored with an infared thermometer immediately upon collection of the seawater sample to be analyzed for additional parameters (e.g. pathogens and Chl a). pH will be monitored using a pH meter (hatchery and laboratory samples) or Hach kit (field samples). Initially concordance between the pH meter and Hach kit will be established prior to use. Dissolved oxygen will be measured *in situ* with a YSI 57 DO meter. Salinity will be measured using a refractometer or via

conductivity measurements from an ion selective electrode and these data will also be used to estimate calcium levels. The latter will also be empirically quantified with an ion selective electrode (InSitu). Chlorophyll a analyses will be conducted 1 L seawater samples after being filtered through Whatman® GF/C filters and extracted with methanol according to the methods of the Standing Committee of Analysts (1980); absorbance will then be read in a Tecan Genios spectrophotometer at 665 and 750 nm and regressed against standards. Alkalinity will be analysed from 50 mL unfiltered seawater samples via titration according to the methods of Mackereth et al. (1978). Suspended particulate organic matter will be quantified according to the methods of Allen (1989).

Animal Maintenance and Quarantine Facility - All oysters used in laboratory trials described above will be transported on ice from the hatchery to the University of Washington's Pathogen Quarantine Facility. This facility is approved by the Washington Department of Fish and Wildlife (WDFW) for examination of selected exotic pathogens (e.g. OsHV) and native pathogens infecting local species or those approved by WDFW (i.e. in possession of an import permit for that species). The Friedman Laboratory at UW renews its importation permits with WDFW annually. All effluent from the quarantine facility is chlorinated at 15 ppm for 24hr and dechlorinated prior to release into the sewer. To reduce aerosol contamination, the circulating air in the facility in UV irradiated 24hr per day. The facility has an antiseptic foot bath for entry and exit from the facility. Only authorized personnel are allowed entry into the facility where they follow strict sanitation protocols. No pathogens are allowed to leave this facility and associated analytical laboratories. No pathogens will be used in any hatchery facility and biosecurity procedures will be in place to prevent any transfer of experimental pathogens to the environment or hatcheries.

7. Participation by persons or groups other than the applicant

In addition to the applicant and colleagues at the University of Washington, several other parties are integrally involved in this project including commercial shellfish producers (Taylor Shellfish Farms, Quilcene, WA; Whiskey Creek Oyster Hatchery, Netarts, OR; Bay Center Mariculture, Willapa Bay, WA.) a private shellfish health laboratory (AquaTechnics, Sequim, WA) and a state governmental agency (Washington Department of Fish and Wildlife: Shellfish Disease, Pest and Predator Control Program). As part of research objective 2 of this proposal; sampling will occur at two hatcheries and three wild sites (two directly corresponding to the hatcheries). Whiskey Creek Oyster Hatchery will provide samples and data from the hatchery and field site in Oregon. Willapa Bay, WA will be also be sampled to characterize factors associated with the natural set. Aquatechnics will be responsible for sampling this site. The third site Dabob Bay, WA will include samples from the field collected by Washington Department of Fish and Wildlife and samples from a commercial hatchery provide by Taylor Shellfish Farms. Sample analysis will be partitioned between the University of Washington and AquaTechnics laboratories.

8. Project management

Steven Roberts is the Principal Investigator of this project and will oversee the project. Project budgetary processes will be managed by the University of Washington's Office of Sponsored Programs. In terms of research activities, Roberts will be responsible for characterizing oyster larvae stress response via development and implementation of the gene expression assay. Using similar approaches Roberts will also be responsible for evaluating physiological changes (eg virulence, growth) in *Vibrio tubiashii* during laboratory trials and also in any hatchery and field samples that are positive for *Vibrio tubiashii*. Carolyn Friedman is a Co-PI on the proposal and will be primarily responsible for overseeing all laboratory trials and, along with Co-PI Ralph Elston will be responsible for characterizing the presence of pathogens in hatchery and field samples. Ralph Elston will coordinate sampling at the Quilcene and Willapa Bay and will be responsible for bacteriological analysis and larval analysis from commercial hatcheries. Dr. Ralph Elston was selectd as a consulting Co-PI because of his extensive experience and recent published data (Elston et al. in press) in regard to the *Vibrio tubiashii* out break in oyster hatcheries. Dr. Elston's laboratory also possess bacterial isolates and antisera developed to identify *V. tubiashii* needed for the study and is recognized by federal agencies (USDA) as a qualified laboratory with approved protocols for diagnostic testing of shellfish disease causing agents.

The following pages contain:

Curriculum vitas for
Steven Roberts (PI)
Ralph Elston (Co-PI)
Carolyn Friedman (Co-PI)

Letters of agreement and support from those providing in-kind contributions;
Ralph Elston, **AquaTechnics** (Co-PI)
Russell Rogers, **Washington Department of Fisheries and Wildlife**
Jonathan Davis, **Taylor Shellfish Farms**

Letters of agreement and support from others directly involved in the proposal;
Sue Cudd, **Whiskey Creek Shellfish Hatchery**
Richard Wilson, **Bay Center Mariculture**

Letter of support;
Robin Downey, **Pacific Shellfish Growers Association**

CURRICULUM VITAE – STEVEN BEYER ROBERTS

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Academic Experience Ph.D. – University of Notre Dame (South Bend, IN) – 2002
Integrative Cell and Molecular Physiology

B.S. – North Carolina State University (Raleigh, NC) – 1997
Natural Resources – Concentration in Marine and Coastal Resources

Professional Experience *2006-Present* · Assistant Professor
University of Washington, Seattle, WA

2006-Present · Adjunct Assistant Scientist
Marine Biological Laboratory, Woods Hole, MA

2003-2006 · Assistant Research Scientist
Marine Biological Laboratory, Woods Hole, MA

Select Publications

Roberts SB, Goetz G, White S, Goetz F (2008) Analysis of genes isolated from plated hemocytes of the Pacific Oyster, *Crassostreas gigas.* Marine Biotechnology. Jul 12. [Epub ahead of print]

Roberts SB, Gueguen Y, de Lorgeril J, Goetz F. (2008) Rapid accumulation of an interleukin 17 homolog transcript in *Crassostrea gigas* hemocytes following bacterial exposure. Developmental and Comparative Immunology. Volume 32, Issue 9, Pages 1099-1104

Roberts, SB (2008) USDA-NRAC Technical Report: "Development of genetic markers to assess disease resistance in the eastern oyster" 29 pages URL: http://tinyurl.com/23c7my

Lyons MM*, Lau Y-T, Carden WE, Ward JE, Roberts SB, Smolowitz R, Vallino J, Allam B. (2007) Characteristics of marine aggregates in shallow-water ecosystems: Implications for disease ecology. EcoHealth. 4, 406–420

Hodgins-Davis A*, Roberts SB, Cowan D, Atema J, Avolio C, Defaveri J, Gerlach G. (2007) Characterization of SSRs from the American lobster, *Homarus americanus.* Molecular Ecology Notes. 7:330-332

Rodgers BD, Roalson EH, Weber GM, Roberts SB, Goetz FW. (2007) A Proposed Nomenclature Consensus for the Myostatin Gene Family. AJP- Endocrinology and Metabolism. 292(2):E371-2

Lyons MM*, Smolowitz R, Dungan C, Roberts SB. (2006) Development of a real-time quantitative PCR assay for the hard clam pathogen, Quahog Parasite Unknown (QPX). Diseases of Aquatic Organisms. 72(1):45-52

Select Publications continued

Roberts SB, Romano C, Gerlach G. (2005) Characterization of EST derived SSRs from the bay scallop, *Argopectens irradians*. Molecular Ecology Notes. 5: 567-568

Biga PR, Roberts SB, Iliev DB, McCauley LA, Moon JS, Collodi P, Goetz FW. (2005) The isolation, characterization, and expression of a novel GDF11 gene and a second myostatin form in zebrafish, Danio rerio. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 141: 218-230

Roberts SB, McCauley LAR, Devlin RH, Goetz FW. (2004) Transgenic salmon over-expressing growth hormone exhibit decreased myostatin transcript and protein expression. Journal of Experimental Biology. 207(Pt 21):3741-8

Kim H-W*, Mykles DL, Goetz FW, Roberts SB. (2004) Characterization of an invertebrate myostatin homologue from the bay scallop, *Argopecten irradians.* BBA – Gene Structure and Expression. 1679(2):174-9

* *indicates student author*

Recent Presentations

Characterizing the response of *Vibrio tubiashii* to changes in environment. Pacific Coast Division, National Shellfisheries Association Conference, October 1-3, 2008. Chelan, WA

Overview of Shellfish Activities at the University of Washington. USDA-WERA099: Broodstock Management, Genetics and Breeding Programs for Molluscan Shellfish. April 6, 2008. Providence, RI

Characterizing disease resistance in native oysters that have experienced disease pressure National Shellfisheries Association Conference. April 6-10, 2008. Providence, RI

Disease tolerance and immune response in oysters. WA Resource Agencies: WA Department of Fish and Wildlife, WA Department of Natural Resources, and WA Department of Ecology. Olympia, WA. March 26, 2008

Immune response in shellfish. USGS Western Fisheries Research Center, Seattle WA. March 4, 2008

Gene expression profiling and cellular characteristics of *Crassostrea virginica* hemocytes: evaluating interactions of physical stress and disease. Aquaculture 2007, San Antonio, TX March 1, 2007

Genomic approaches in characterizing shellfish disease: interrelationships between animal, human and ecosystem health. Cummings School of Veterinary Medicine at Tufts University, Annual Symposium: Marine and Aquatic Medicine & Conservation. North Grafton, MA. April 22, 2006

Courses Taught

FISH310: Biology of Shellfishes
FISH507: Bioinformatic Approaches in Fisheries Science
FISH510: Innovations in Molecular Techniques
FISH414: Integrative Environmental Physiology (Winter 09)

Curriculum vita RALPH A. ELSTON

Chief Scientist, AquaTechnics Inc. 455 West Bell Street, Sequim, WA 98382 USA
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Education:

Ph.D. Veterinary Pathology (1980), Cornell University
M.S. Ecology (1975), University of California, Davis
B.S. Fisheries (1973), University of California, Davis

Positions Held:

Chief Scientist and President, AquaTechnics 1995 to present
Board of Directors, Pacific Shellfish Institute, 2006-present

Project Manager (part time), Pacific Shellfish Institute, 1996-2006
Affiliate Professor, University of Washington, 1988-present
Senior Research Scientist and Staff Scientist, Battelle Marine Sciences Laboratory 1982-1995
Research Associate, Cornell University, 1980-1981

Professional Memberships:

National Shellfisheries Association, Pacific Coast Section of the National Shellfisheries Association, World Aquaculture Society, Fish Health Section of the American Fisheries Society, Society for Invertebrate Pathology, European Association of Fish Pathologists.

Selected Peer Reviewed Publications for total of approximately 80 (primarily on topics concerning shellfish infectious diseases and health management):

Elston, R., Häse, C., Hasegawa, H., Humphrey, K., Polyak, I. in press. Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and management. Diseases of Aquatic Organisms.

Elston, R. A., E.W. Cake, Jr., K. Humphrey, W. Isphording and J.E. (Jack) Rensel. 2005. Dioxin and heavy metal contamination of shellfish and sediments in St. Louis Bay, Mississippi and adjacent marine waters. Journal of Shellfish Research 24(1): 227-241.

Friedman, C. S., Estes, R. M., Stokes, N.A., Burge, C.A., Hargorve, J. S., Barber, B. J., Elston, R. A., Burreson, E. and Reece, K. S. 2004. Herpes-like virus detected in juvenile Pacific oysters, *Crassostrea gigas* Thunberg, in Tomales Bay, California, may be related to a mortality episode. Diseases of Aquatic Organisms. Diseases of Aquatic Organisms 63(1): 33-41.

Estes, R. M., Friedman, C. S, Elston, R. A. and Herwig, R. P. 2004. Pathogenicity testing of shellfish hatchery bacterial isolates on Pacific oyster (*Crassostrea gigas*) larvae. Diseases of Aquatic Organisms 58: 223-230.

Elston, R. A., Dungan, C. F., Meyers, T. R., Reece, K. 2003. *Perkinsus* sp. infection risk for manila clams, *Venerupis philippinarum*, (A. Adams and Reeve, 1850) on the Pacific coast of north and central America. Journal of Shellfish Research 22(3): 667-674.

Elston, R. A., Cheney, D., MacDonald, B., Suhrbier, A. 2003. Tolerance and response of manila clams , *Venerupis philippinarum*, (A. Adams and Reeve, 1850) to low salinity. Journal of Shellfish Research 22(3): 661-666.

Elston, R. and War, J. 2003. Biosecurity and health management for intensive mollusc culture. Pages 157-170 *in* C.-S. Lee and P. J. O'Bryen, editors. Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables. The World Aquaculture Society, Baton Rouge, Louisiana.

Book:

Elston, R. A. 1999. Health Management, Development and Histology of Seed Oysters. 120 pp. World Aquaculture Society, Baton Rouge, LA. 110 pages.

CURRICULUM VITAE – CAROLYN S. FRIEDMAN

School of Aquatic and Fishery Sciences
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Education:
Ph.D. Comparative Pathology (1990), University of California, Davis
B.A. Aquatic Biology (1983), University of California, Santa Barbara

Positions Held: Associate Professor, School of Aquatic & Fishery Sciences, University of Washington 2004 to present. Assistant Professor, School of Aquatic and Fishery Sciences, University of Washington 2001-04. Senior Fish Pathologist, California Department of Fish and Game, 1998 to 2002. Associate Fish Pathologist, California Department of Fish and Game, 1988 to 1998.

Awards and Honors:

- J.A. Valentine Visiting Professor, University of Otago, New Zealand 1995.
- American Fisheries Society, Most significant paper in the Journal of Aquatic Animal Health award in 2001.
- College of Ocean and Fishery Sciences, University of Washington, Distinguished Research Award, 2007.
- Chercher Etranger, Universite de Bretagne Occidentale, CNRS, Brest France – September 25, 2007-January 3, 2008

Selected Publications: (Out of 60)

Travers, M.A., Le Bouffant, R., Friedman, C.S., Buzin, F., Cougard, B., Huchette, S., Koken, M., Paillard, C. 2008. Pathogenic *Vibrio harveyi*, in contrast to non-pathogenic isolates, intervenes with the p38 MAPK pathway to avoid an abalone haemocyte immune response. Journal of Cellular biochemistry. In press.

Wight, N., Suzuki, J, Vadopalas, B. and Friedman, C.S. 2008. Development and optimization of quantitative PCR assays to aid *Ostrea conchaphila* restoration efforts. Journal of Shellfish Research (Accepted after revision).

Straus, K.M., and Friedman, C.S. In review. Restoration aquaculture of the pinto abalone (*Haliotis kamtschatkana kamtschatkana* Jonas): impacts of rearing method on behavior, growth, and survivorship. Marine and Freshwater Research.

Rothaus, D.P., Vadopalas, B., and Friedman, C.S. 2008. Precipitous declines in pinto abalone (*Haliotis kamtschatkana kamtschatkana*) abundance in the San Juan Archipelago, Washington, USA, despite statewide fishery closure. Canadian Journal of Fisheries and Aquatic Sciences. In press.

Burge, C.A., Judah, L.R.J., Conquest, L, Griffin, F.J., Cherr, G.N., Cheney, D., Suhrbier, A, Suhrbier, A, Olin, P.G., and Friedman, C.S. 2007. Examination of factors affecting survival of the Pacific oyster, *Crassostrea gigas* along the west coast of North America: Multiple stressors, family lines and seasonality. Journal of Shellfish Research. 26(1):163-172.

Chambers, Melinda D., Glenn R. VanBlaricom, Lorenz Hauser, Fred Utter, Carolyn S. Friedman. 2006. Genetic structure of black abalone (*Haliotis cracherodii*) populations in the California islands and central California coast: impacts of larval dispersal and decimation from Withering Syndrome. Journal of Experimental Marine Biology and Ecology 331:173-185.

Vadopalas, B. Bouma, J., Jackels, C., and Friedman, C.S. 2006. Application of quantitative PCR for simultaneous identification and quantification of larval abalone Journal of Experimental Marine Biology and Ecology.

Burge, C.A., Griffin, F.J., and Friedman, C.S. 2006. Summer mortality and herpes virus infections of the Pacific oyster, *Crassostrea gigas*, in Tomales Bay, California. Diseases of Aquatic Organisms.

Friedman, C.S., Brown, H.M, Ewing, T.W., Griffin, F.J., Cherr, G.N. 2005.
Pilot study of the Olympia oyster *Ostrea conchaphila* in the San Francisco Bay Estuary: description and distribution of diseases. Diseases of Aquatic Organisms 65:1-8.

L.I. Vilchis, M.J. Tegner, J.D. Moore, C.S. Friedman, K.L. Riser, T.T, Robbins, and P.K. Dayton. 2005. Effects of ocean warming on the growth, reproduction and survivorship of red and green abalones in southern California. Ecological Applications 15(2):469-480.

Friedman, C.S., Stokes, N.A., Burreson, E.S., Barber, B., Elston, R.A. and Reece, K. 2005.
Identification of a herpes-like virus in Pacific oysters, *Crassostrea gigas*
Thunberg, in Tomales Bay, California. Diseases of Aquatic Organisms 63:33-41.

Braid, B. A., J. D. Moore, T. T. Robbins, R. P. Hedrick, R. S. Tjeerdema, and C. S. Friedman. Health and survival of red abalone, *Haliotis rufescens*, under varying temperature, food supply, and exposure to the agent of withering syndrome. Journal of Invertebrate. Pathology.

Estes, R.M., Friedman, C.S., Elston, R.A., and Herwig, R. 2004. Pathogenicity of shellfish hatchery bacterial isolates to Pacific oyster larvae, *Crassostrea gigas*: A microplate assay. Diseases of Aquatic Organisms 58:223-230.

Luengen, A.C., Friedman, C.S., Raimondi, P.T., and Flegal, A.R. 2004. Evaluation of Immune Responses as Indicators of Contamination in San Francisco Bay, Using a Novel Phagocytosis and Phagocytic Index Method Developed for Mussels. Marine Environmental Research. 57(3): 197-212.

Friedman, C.S., and Finley, C.A. 2003. Evidence for an anthropogenic introduction of "Candidatus *Xenohaliotis californiensis*", the etiological agent of withering syndrome, into northern California abalone populations via conservation efforts. Canadian Journal of Fisheries and Aquatic Sciences 60:1424-1431.

Friedman, C.S., Trevelyan, G., Mulder, E.P., and Fields, R. 2003. Development of an oral administration of oxytetracycline to control losses due to withering syndrome in cultured red abalone *Haliotis rufescens*. Aquaculture 224(1-4):1-23.

Friedman, C.S., Biggs, W, Shields, J.D. and Hedrick, R.P. 2002. Transmission of Withering Syndrome in black abalone, *Haliotis cracherodii* Leach. Journal of Shellfish Research. 21(2):817-824.

AquaTechnics

October 20, 2008

Dr. Carolyn Friedman
Associate Professor
School of Aquatic and Fisheries Sciences
University of Washington
Box 355020
Seattle, WA 98105

Dear Dr. Friedman:

In regard to the proposal you and Dr. Roberts are preparing to submit to the N.O.A.A. Saltonstall-Kennedy program entitled: THREATS TO BIVALVE AQUACULTURE AND FISHERIES: THE INFLUENCE OF EMERGING DISEASES AND ENVIRONMENTAL CHANGE, I am pleased to participate on this project as a co-investigator and also to contribute in kind effort to the project.

I can provide matching funds in year one in the amount of $12,900.and in year 2 of the project in the amount of $13,287. This in-kind contribution is based on my fully burdened charge rate of $75. per hour for a total of 172 hours in each year.

Time charged to the project by myself and staff would be at fully burdened rates of $75. per hour for myself and $33. per hour for laboratory specialist Ildiko Polyak.

Thank you for the opportunity to participate in this important project.

Sincerely,

Ralph Elston, PhD

AquaTechnics*, P.O. Box 687, Carlsborg, WA 98324 USA Tel: 360-681-3122 Fax: 360-681-3123
offices located at 455 W. Bell Street, Sequim, Washington 98382
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State of Washington
Department of Fish and Wildlife

Point Whitney Shellfish Lab, 1000 Point Whitney Road, Brinnon WA 98320
Telephone (360) 586-1498; TDD (360) 902-2207; Fax (360) 586-8408

16 October 2008

Dr. Carolyn Friedman
School of Aquatic and Fisheries Sciences
University of Washington
PO Box 355020
Seattle WA 98195

Re: THREATS TO BIVALVE AQUACULTURE AND FISHERIES: THE INFLUENCE OF EMERGING DISEASES AND OCEAN ACIDIFICATION

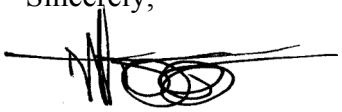
Achieving healthy, diverse and sustainable fish and wildlife populations and their supporting habitats is part of the core mission of the Washington Department of Fish and Wildlife. The Shellfish Disease, Pest and Predator Control Program can make an in-kind contribution the study noted above in the form of up to 20 days (one month) of staff time for a Fish and Wildlife Biologist 3, including benefits and travel cost. This staff position can assist in field, lab, or office work.

Here is the cost estimate for this in-kind contribution;

F&W Biologist 3, one month	4770.00
Benefits	1459.00
Indirect cost (25.67%)	1611.00
WDFW vehicle and fuel	2000.00
Total	**9840.00**

We look forward to working with you on this important project. If you have any questions, please feel free to call me at anytime.

Sincerely,



Russell E. Rogers, Jr.
Fish and Wildlife Biologist
Shellfish Disease, Pest, and Predator Control Program
Washington Department of Fish and Wildlife

2008701 Broad Spit Road
Quilcene, WA 98376
USA
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October 24, 2008

Steven Roberts
School of Aquatic and Fishery Sciences
University of Washington
1122 NE Boat Street
Seattle, WA 98105

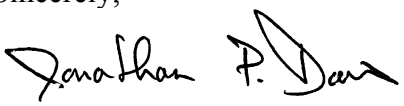
Dear Dr. Roberts:

I am writing to express my support for your proposal submitted to the Saltonstall Kennedy Program entitled, "THREATS TO BIVALVE AQUACULTURE AND FISHERIES: THE INFLUENCE OF EMERGING DISEASES AND ENVIRONMENTAL CHANGE". I note that Drs. Ralph Elston and Carolyn Friedman are co-PI's on this proposed research. This is an extremely timely project that would positively impact our shellfish industry in the Pacific Northwest. As you know, we have had unprecedented losses of shellfish larvae and seed due to the dual effects of *Vibrio tubiashii* and abnormal environmental conditions, including significantly lower pH in surface waters that appear to affect marine bivalves quite negatively. Your proposed studies to undertake a series of laboratory studies and environmental sampling in the hatcheries and surrounding water bodies appears sound and that the information gained will result in important changes in rearing technology and techniques that will enable the shellfish industry to move forward. Time is of the essence as the industry has had several, successive poor production years, especially with respect to the production of Pacific oysters. Taylor Shellfish Farms has shellfish production sites throughout Puget Sound and extensive beds in Willapa Bay on the Washington coast. We maintain hatchery facilities in Dabob Bay, Kona, Hawaii and an experimental facility in Port Gamble, Washington. The main production facility on Dabob Bay has experienced significant losses in terms of oyster production capability over the last three years.

To this end, we at Taylor Resources, Inc. can commit in-kind support and provide Pacific oyster larvae as needed over the course of the proposed studies. These include small batches of larvae as needed and 4 dedicated spawns in the first year of the study and an additional 3 dedicated spawns in the second year of the study. Provision of limited numbers of larvae on an "as needed" basis in each year of the study have a value of $1000 per year or $2000 over the two year study. Dedicated spawns have an in-kind value of $1500 each for a total of $7500 over the duration of the project. Total in-kind support over the two year project would total $9500.

Please let me know how we can help further. We are very hopeful the information gained form this research will allow us to better predict mortality events, improve hatchery practices, and develop improved broodstock.

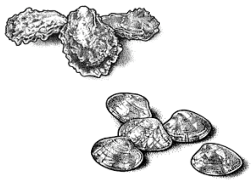
Sincerely,



Jonathan P. Davis, Ph.D.

Research and Development
Taylor Resources, Inc.

Whiskey Creek Shellfish



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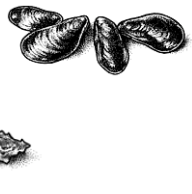

NOAA Saltonstall-Kennedy program

To Whom it may concern:

This letter is to express my support for the grant proposal titled "THREATS TO BIVALVE AQUACULTURE AND FISHERIES: THE INFLUENCE OF EMERGING DISEASES AND ENVIRONMENTAL CHANGE" with the lead PI as Dr. Steven Roberts and Co-PIs of Drs Ralph Elston and Carolyn Friedman.

This research is critical for our survival as a shellfish hatchery that supplies more than 50% of the shellfish industry on the west coast with their larvae. For the last two years our production has been reduced by as much as 80% to the point that we are unable to sustain our business, having a significant impact on the shellfish industry as a whole.

The grant proposal plans to look at single and multiple stressors (pH, temperature, pathogens: Vibrio tubiahsii and OsHV on larval oyster survival. They will compare early to late phase larvae and may also look at early seed. They also plan to follow larval cultures at Taylors and Whiskey Creek (3-4 per year) and take samples for Vt assessment in water, with oysters, and with plankton. We will be collaborating by following spawns and conducting specific spawns to assess the influence of environmental parameters on larval survival and taking environmental samples.

Water quality parameters (pH, temp, DO, ammonia, alkalinity and hopefully calcium) will also be monitored as well as growth and survival data as well. In parallel, growers will take water samples from adjacent bays and do the same assessments but also enumerate bivalve larval density.

Again, I strongly encourage you to fund this research as it may be critical to solving these problems and the ultimate survival of the West Coast Shellfish Industry. The entire industry agrees that this is the most critical problem we are currently facing.

Sincerely,

Sue Cudd
Owner/Manager
Whiskey Creek Shellfish Hatchery, Inc.

To: Steven Roberts and Carolyn Friedman

RE: Funding support, NOAA - SK proposal to address natural and cultured loss of bivalve mollusks

Our industry has experienced three seasons of negative impacts on oyster larval survival and seed production. The loss of larval shellfish whether from the hatchery systems in 2006, 2007 and 2008 or the important natural sets in places such as here in Willapa have had a significant impact of oyster crop inventories on our farms.

Key suspects as a causal agents in this phenomenon are a bacterium Vibrio tubiashii and in California an oyster herpes virus. In response to this situation key research proposals have been proposed which could go far to address the interaction between environmental factors, microorganisms and the larval mortalities. I understand the study will require characterization of ocean water and microbiological analysis from shellfish growing areas. In conjunction, to me, an important factor would be the testing and laboratory confirmation of how changes in specific environmental factors such as sea water temperature and pH may promote the virulence of such microorganisms. Industry support could be incorporated, for example we could also help in sample collection from our location at Bay Center on Willapa Bay.

Thus, I am writing to support specifically the proposal by Steven Roberts and Carolyn Friedman presented to the NOAA - SK program to investigate virulence of such microorganisms. I also encourage the funding of other associated proposals investigating causes of these larval failures and support Dr. Ralph Elston who has indicated a supporting role between those working on this very critical problem.

Thanks in advance,

Dick

Richard L. Wilson, Ph.D.
President Bay Center Mariculture
Board Member Pacific Coast Shellfish Growers Assoc.
www.baycenterfarms.com
360-875-6172
October 24, 2008

Pacific Coast
SHELLFISH GROWERS ASSOCIATION
Partners with Mother Nature

sustainably farmed
oysters, clams, mussels, scallops

October 28, 2008

Dr. Steven Roberts
School of Aquatic and Fishery Sciences
University of Washington
Fisheries Research and Teaching Building
Room # 209
1140 NE Boat Street
Seattle, WA 98105

Dear Dr. Roberts,

The Pacific Coast Shellfish Growers Association strongly supports your research proposal, "Threats to Bivalve Aquaculture and Fisheries: The Influence of Emerging Diseases and Environmental Change" you are submitting to the Saltonstall-Kennedy Program of NOAA.

Your examination of the several environmental issues that appear to be contributing to larval and young shellfish seed mortality is badly needed if we hope to find solutions in a timely manner. The focus on links between seed set and the presence of Vibrio tubiashii, pH, temperature and the herpes virus, both in hatchery produced and wild set seed, is imperative to our gaining a better understanding of the dynamics at play.

As you are aware, two of the largest hatcheries here on the West Coast have experienced serious mortality issues in the past two years, leading to insufficient seed availability for shellfish growers here on the West Coast. Growers that rely on hatchery seed as well as growers who are still collecting wild set seed for their farms are being impacted and the economic consequences are already being felt.

We further support the collaborative approach proposed for this research, including Drs. Ralph Elston, Carolyn Friedman, and hatchery personnel involved in the various facets of this project.

Thank you for your interest in this critical subject. Please contact me if there is anything the shellfish growers can do to help facilitate this research.

Sincerely,

Robin Downey

Robin Downey
Executive Director

Olympia: 120 State Ave., NE PMB #142, Olympia, WA 98501 phone: 360-754-2744 fax: 360-754-2743
Port Townsend: 2023 E. Sims Way #235, Port Townsend, WA 98368 phone: 360-379-9041 cell: 360-870-9611
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