Ocean Acidification

Increasing atmospheric CO_2 is clearly one of greatest environmental challenges our society will face. Atmospheric CO_2 levels (currently ~384 ppm; Solomon et al 2007) have increased by more than 100 ppm since the beginning of the industrial revolution (Sabine et al 2008) and are expected to double that of pre-industrial levels by 2050 (Feely et al 2004). This unprecedented increase in atmospheric CO_2 is mitigated to some degree by oceanic uptake of CO_2, which can account for almost a third of the anthropogenic carbon added to the atmosphere (Sabine & Feely 2007, Sabine 2004). Thus, the ocean has served as a primary reservoir for excess atmospheric CO_2 and played an essential role in tempering climate change. However, mounting research has revealed that the magnitude of CO_2 being absorbed by the ocean is beginning to manifest globally as chemical changes in seawater that increase CO_2 partial pressure (pCO_2) and decrease pH (Rost et al. 2008, Falkowski et al. 2000). Briefly, concentrations of dissolved CO_2, bicarbonate and hydrogen ions are maintained in equilibrium in seawater and enable the ocean to buffer against net changes in pH. However, excess dissolved atmospheric CO_2 has begun to disrupt this equilibrium, resulting in a net increase in hydrogen ion concentration which results in decreased seawater pH ($pH = -\log_{10}[H^+]$). A decrease in pH also represents a decrease in carbonate ion ($CaCO_3^{-2}$) concentrations, as well as the saturation states of both argonite and calcite. Argonite and calcite are biogenic minerals essential to shell and skeleton formation in calcareous organisms such as bivalves and corals. Taken together, these compositional changes in seawater chemistry are referred to as ocean acidification. Model calculations estimate that since pre-industrial times, pH has decreased by 0.1 units, representing an approximate 30% increase in seawater acidity (Haugan & Drange 1996). It is predicated that by 2100 pH is likely to fall 0.3-0.5 units (Zeebe and Wolf-Gladrow 2001, Caldeira and Wickett 2005).

To date, much of the work on the biological impacts of ocean acidification has focused on the calcification process (De'Ath, et al. 2009, Beesley et al., 2008, Moy et al. 2009). Recent work has measured a 14% decrease in coral reef calcification rates since 1990 (De'Ath, et al. 2009) and has demonstrated a strong correlation between higher atmospheric CO_2 and lowered shell weight in planktonic foraminifera (Moy et al. 2009). In addition, CO_2 induced changes in pH have been shown to significantly reduce the health of the mussel *Mytilus edulis* (Beesley et al., 2008). While these results suggest great physiological stress beyond calcification, we know much less about the general physiological responses of organisms to ocean acidification, an area identified by NOAA as a key to the development of indices to track ecosystem responses.

There are also limited studies that have examined the effects of ocean acidification along in conjunction with other stressors. Of the few studies examining multiple stressors, one examined the effect of elevated pCO_2 and thermal stress in sea urchin larvae (O'Donnell et al 2008). Researchers found that the ability of sea urchin larvae to mount a physiological response mitigating acute thermal stress was compromised under elevated CO_2 conditions (O'Donnell et al 2008). This is in agreement with a previous study where crabs exposed to elevated pCO_2 were more susceptible to high temperature mortality (Metzger, 2007). While it makes sense to examine secondary stressors such as temperature, as elevated pCO_2 will also effect climate, it is likely pCO_2 induced environmental changes will render marine organisms more susceptible to other stressors such as pollution and disease.

Finally, we know relatively little about how the ecology and activity of planktonic or host-associated microbes may respond to ocean acidification. However, recent work in microbial ecology has identified pH as an important predictor of microbial community composition in soil. Changes in soil and freshwater pH were well correlated with changes in both overall microbial community composition and that of important functional groups (e.g., Jones et al. 2009, Fierer et al. 2006). These results suggest that lowered ocean pH may also be accompanied by changes in aquatic microbial community composition. Changes in marine microbial community composition or function could have significant direct and indirect impacts on other marine organisms and ecosystem health as a whole, as microbes mediate many important biogeochemical cycles, including cycling of major nutrients. As pathogens, they also may play a direct role in the population dynamics of other taxa including fish, corals, and bivalves.

This proposal will use an integrated approach to assess the potential ecosystem-level effects of ocean acidification by examining the *interactions among elevated CO_2, oyster physiology and the host-associated microbial community*. Understanding these impacts of ocean acidification has implications for population and community level responses of different taxa that could have ecosystem-level implications. In particular, we will address the following questions:

Q1) How will elevated pCO2 directly impact oyster physiology?
Q2) How will elevated pCO2 impact microbial community structure?
Q3) What are the indirect effects of ocean acidification on oysters?

In what follows, we discuss what is known about the impact of ocean acidification on oysters, microbes, and their biological interactions.

IMPACTS OF OCEAN ACIDIFICATION ON SHELLFISH

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). Nitrogen, phosphorus and other nutrients that are not incorporated into oyster tissue are excreted and can be utilized by keystone estuarine plants such as eelgrass (Newell 2004). Oysters commonly grow in aggregations, and oyster beds have been shown to support high levels of biodiversity (Ferraro and Cole 2007). They engineer habitat integral not only for larval oysters, but for other organisms such as worms, snails, crabs, fish and birds.

The effects of ocean acidification on shellfish calcification rates can vary across species and environments (Doney et al 2009). In general, ocean acidification leads to less calcium carbonate available for shell deposition and in some instance shell dissolution can occur. One of several studies that have examined shell formation found that when mussels and the Pacific oysters were grown at ~740ppm CO_2 in the lab, calcification rates decreased by 25% and 10%, respectively (Gazeau et al 2007).

However, the effects of decreased pH on shellfish are not limited to impacts on calcification, and this has been an area of inquiry since long before ocean acidification was recognized as a global concern. While studying the eastern oyster, Prytherch (1928) concluded that low pH inhibited spawning. Conversely, researchers determined that decreased pH (~-0.35 pH units) had no effect on oyster sperm swimming speed, sperm motility, and fertilization kinetics (Havenhand and Schlegel, 2009). Loosanoff and Tommers (1947) observed decreased filtration in adult oysters at pH below 7.0. Calabrese and Davis (1966) carried out a study examining the pH tolerance of eastern oyster larvae and observed low pH had a negative impact on ciliary action. Here we propose to examine the impact of decreased pH on the physiology of adult oysters to gain insight into potential individual and subsequently population level impacts. In the proposed research we will use a global approach to broadly characterize effects on multiple processes.

IMPACTS OF OCEAN ACIDIFICATION ON MICROBES

Microbes are the most abundant, diverse (Whitman et al. 1998, Venter et al. 2004) and potentially the most important marine organisms as they mediate crucial biogeochemical cycling (Bell et al. 2005, Karl 2007, McGrady-Steed et al. 1997) and play a significant role in marine disease (Harvell et al. 1999, Paillard et al. 2004, Cervino et al. 2008). Marine phytoplankton are responsible for the majority of marine primary productivity and a significant amount of nitrogen fixation, while bacteria and

archaea are responsible for regenerating nutrients (Karl 2007). Impacts of ocean acidification on dissolved organic matter as well as on biogeochemical dynamics of calcium carbonate, organic carbon, nitrogen and phosphorus in the ocean will likely impact the composition and function of microbial communities (Boyd and Doney 2002, Doney et al. 2009). Thus understanding how marine microbes respond to changes in ocean acidification may have important implications for our understanding of marine ecosystem function and large scale nutrient cycling.

Microbial community dynamics are controlled by numerous environmental factors. However, we do know that pH is often a correlate of bacterial diversity and community composition in a range of ecosystems (Zeng et al 2009, Anderson-Glenna et al 2008, Fierer and Jackson 2006, among others). This relationship may result from differences among community members in optimum pH and tolerance to variation in pH that result in differential growth and/or metabolic rates as pH decreases and CO_2 increases. For example, in a lab experiment assessing increased temperature and increased CO_2 on marine cyanobacteria, *Prochlorococcus* exhibited minimal changes, while another cyanobacterial taxa, *Synechococcus,* exhibited increased photosynthesis rates and a 20% increase in cellular Carbon:Phosphorous and Nitrogen:Phosphorous ratios (both phytoplankton species are major components of the open ocean microbial community) (Fu et al 2007).

Changes in environmental conditions can also have an indirect effect on species composition. A recent mesocosm experiment showed that the community structure of free-living marine bacteria changed with increasing pCO_2 (Allgaier et al 2008). They also showed that bacterial activity and dynamics were linked to changes in the phytoplankton community and thus may be indirectly influenced by changes in pCO_2. Similarly, changes in pCO_2, and thus associated changes in pH, will likely also impact the virioplankton community (e.g. see Larsen et al 2008). Changes in the viral component of the marine microbial community likely will have impacts on the bacterial community as well. These few examples, to date, illustrate the potentially complex ecological interactions that will likely be involved in unraveling the impact of ocean acidification on marine communities. Here we propose to examine the impact of increased pH on the planktonic as well as oyster associated microbial community. Changes in the planktonic community will give us a window into the potential for ecosystem and functional impacts. The focus of the work, however, lies in understanding the interaction among changes in microbial community composition, oyster physiology and oyster susceptibility to a pathogen.

OYSTER SYSTEMS: MICROBE DYNAMICS

Microbial communities both in the surrounding water column and in the host tissue, can also have a significant impact on the health of their host and thus on the larger ecosystem by influencing the population dynamics of their host. Changes in the composition, abundance and activity of microbial communities (with respect to both pathogenic and non-pathogenic microbes), can play a role in disease. The importance of molluscan microflora to overall organismal health is analogous that of humans and their microflora (Hickman 2003, Ouwehand et al. 2002). For example, it is becoming clear that human-microbe mutualisms play an important role in human health and that ecological changes altering the relationship can result in disease (Bik et al. 2006, Dethlesthen 2007).

Global climate change has been linked to change in the incidence and distribution of infectious disease in a variety of systems as well as changes in disease dynamics (Lafferty 2009, Harvell et al 2002, Bally and Garrabou 2007). We expect that the impacts of ocean acidification on individual microbial pathogens and on host-pathogen dynamics are likely complex. Changing environmental conditions may provide opportunities for the relative abundance of a pathogen to change and for the geographic distribution of a pathogen to shift or expand. Concurrently, physiological stress associated with ocean acidification conditions may make the host more vulnerable for colonization and infection. For example, changes in the incidence of Cholera, caused by the bacterium *Vibrio cholerae*, have been attributed to the *V. cholerae* population response to climate change related environmental conditions, including temperature, salinity and the presence of copepods, among other factors (Lipp et al 2002). Beyond the direct impact of ocean acidification on microbes, it is very important to consider how the alteration of environmental conditions could impact the host and the host's susceptibility to pathogens. Here we examine the impact of multiple stressors on an oyster to examine the role that pH induced changes in the oyster's resident microbial community may play in the oyster's susceptibility to an introduced pathogen.

Objectives

The overall purpose of this proposal is to better understand the effects of ocean acidification on oyster physiology, microbial community composition and oyster susceptibility to disease. We propose an integrated approach to assess the potential ecosystem-level effects of ocean acidification by examining the *interactions among elevated CO_2, oyster physiology and the host-associated microbial community*. Understanding these impacts of ocean acidification has implications for population and community level responses of different taxa that could have ecosystem-level implications, and, more specifically, will contribute to our understanding of the physiological response of oysters to a high CO_2 world. In addition, this work will offer important insight into the largely unanswered question of how ocean acidification will impact microbial communities. Finally, we will thus examine the impact of multiple stressors on an oyster to understand the role that pH induced changes in the oyster's resident microbial community may play in the oyster's susceptibility to an introduced pathogen. This work will thus give insight into the relationship between pathogen and host, to controls on pathogenicity and on host susceptibility in response to high CO_2, all of which may aid us in anticipating the impact of continued ocean acidification in coastal environment. The methodologies proposed here (see next section for details) will enable us to assess biological impacts in an integrative manner.

We propose to use a mesocosm study to expose oysters and associated natural microbial communities to two elevated pCO_2 conditions (thus lowering pH) in addition to ambient CO_2/pH conditions. Global approaches will be used to assess 1) physiological changes in oysters using next generation sequencing - clonal single molecule array, and 2) microbial community dynamics using DNA fingerprinting techniques (ARISA) (see Approach and Methodology for details). The specific objectives of this proposal are to:

1) ***Characterize the physiological response of oysters to elevated pCO_2 using a transcriptomic approach.***
2) ***Determine how elevated pCO_2 alters microbial composition in the water column and within oyster hosts.***
3) ***Characterize the role of elevated pCO_2 and the host-associated microbial community on an oyster's response to a secondary stressor, an introduced pathogen.***

Approach and Methodology

Experimental Design

In order to complete our research objectives, we will conduct a series of small mesocosm experiments to assess the response of oysters and their associated microbial community to decreased pH induced by elevated pCO_2 levels, such as are expected in the world's oceans in the near future. Specifically, three different scenarios will be examined – current conditions (i) ~380 ppm, and increased levels of CO_2 predicted by the IPCC for 2100 - (ii) 540 ppm and (iii) 970 ppm. Atmospheric CO_2 levels of 540 ppm represent an optimistic scenario from the IPCC report (2001), while CO_2 levels of 970 ppm represents the scenario if we maintain the current CO_2 emissions trajectory (IPCC 2001).

The experiments will be conducted at the Marine Biological Laboratory in Woods Hole, MA in the Marine Resources Center (see attached letter of support). The facility will utilize a system to pump raw seawater in to holding tanks. Thus, the water will be at ambient temperature(s) and will be maintained by the perpetual pumping of fresh seawater into the holding tanks. Oysters will be allowed to acclimate to the system in a large holding tank for a minimum of 5 weeks before treatments are initiated. Oysters and natural microbial communities will then be split into three, separate holding tanks subjected to the three treatments of (i) ~380 ppm, (ii) 540 ppm, (iii) 970 ppm pCO_2 (Figure 1). Commercially supplied gas will be used to treat systems ii and iii, while ambient air will be used for the reference system (~380 ppm). Oysters and associated microbial communities will be subjected to each CO_2 treatment for a period of 5 weeks.

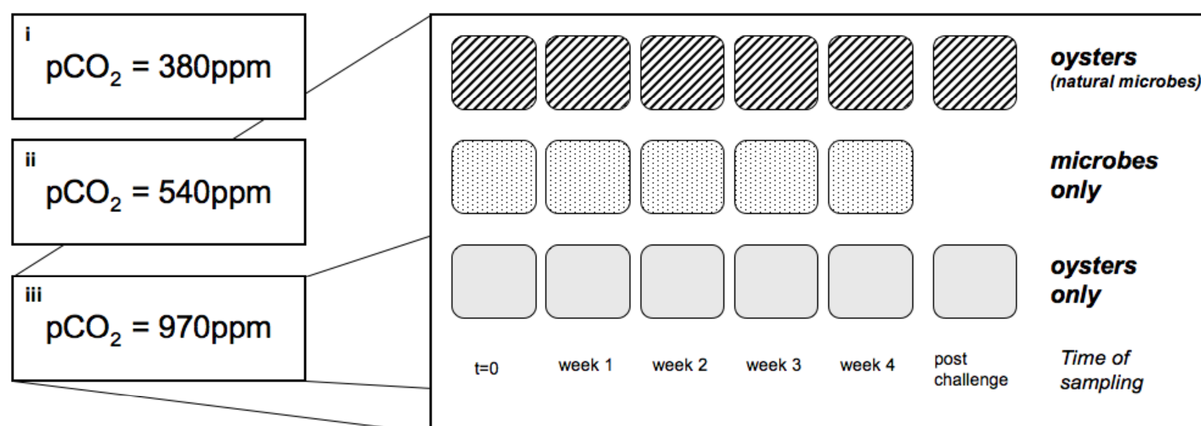

Figure 1. Experimental Design. Three CO_2 treatments will be used including ambient (380ppm) and two elevated levels corresponding to predicted levels for 2100. Each treatment will contain 17 small culture vessels (SCVs) (at right).

Within each of these three systems will be a series of small culture vessels (SCVs). The SCVs will contain one of three biotic sample groups: 1) ***oysters with natural resident and planktonic microbial community*** 2) ***natural planktonic microbe community only (no oysters)***, and 3) ***oysters only***. Sample groups that include oysters will have 6 oysters in each SCV. The third group (oysters only) will be treated with asulfadimethoxine-ormetoprim and norfloxacin solution in a separate, sterile container for 3 hours to eliminate the natural microbial community (Boettcher et al 1999) prior to transfer to the SCVs. Groups one and two will also be transferred to separate, sterile containers without antibiotics for the same duration, prior to transfer to their respective SCVs.

The enlarged system diagram on the right of Figure 1 illustrates the setup of the SCVs within each pCO_2-treated holding tank. Individual SCVs will be enclosed with 2um nylon mesh to isolate microbial communities from the larger treatment tank such that each SCV is an independent unit within its pCO_2 treatment. Ambient seawater will be used for the three treatment systems and fed by header tanks where CO_2 is added. Flow in the systems (i-iii) will be minimal to maintain CO_2 and temperature. pH will be recorded 3 times a day throughout the experiment. Oysters will be fed non-viable, mixed algae shellfish diet (Shellfish Diet; Reed Mariculture).

During the final week of the experiment, each remaining SCV containing oysters will be subjected to a pathogen exposure (*V. tubiashii*). *V. tubiashii* will be grown over night in 1x Luria Bertani broth plus 1% NaCl at 25C, 250RPM. The following day, the bacteria will be pelleted, resuspended and added to the SCVs at a concentration of approximately 3 x 10^4 CFU/mL. Prior to the start of the proposed project we will empirically determine the dose that is likely to cause mortality on the order of days to weeks. All effluent from systems will be treated to sterilize the water.

Sampling
Entire SCVs will be sampled for analysis weekly (t=0, week 1, week 2, week 3, week 4). Each week a total of 36 oyster will be sampled. For each of the three pCO_2 treatments, 6 oysters with natural microbes and 6 oysters devoid of microbes will be sampled (12 x 3=36). Oysters will be processed one at a time. They will be shucked with a sterile oyster knife, gill tissue will be removed using sterile forceps and immediately frozen on dry ice. At the completion of tissue collections, the tubes will be stored at -80C until they are processed.

At the end of week 4, only 2 SCVs will remain in each of the three pCO_2 treatments. The oysters from these SCVs will be subjected to a pathogen challenge. Oysters will be observed and mortalities documented over a period of 1-2 weeks during *V. tubiashii* exposure. To collect samples of microbial communities, 300ml of water from every SCV will be filtered in duplicate onto a 0.2um filter followed with 3ml preservation buffer (10mM Tris pH 8.0, 100mM EDTA, 0.5M NaCl). The filter will be placed in a sterile 50mL conical tube and frozen at -80ºC until further analysis.

In what follows, we discuss the specific methods that will be used to characterize effects of elevated pCO_2 on (1) oyster physiology, (2) microbial community composition, and (3) the ability of oysters to deal with a secondary stressor.

Oyster physiological response

To characterize the physiological response of adult oysters to changes in pCO_2, we will examine differences in gene expression patterns in oysters from the experiment described above. At t=0 and for each successive week, oysters will be harvested and gill tissue collected for RNA isolation. In order to examine how elevated pCO_2 impacts adult oyster physiological and provide insight on the implications of elevated CO_2, a two-phase approach will be taken. Firstly high-throughput short-read sequencing technology (also known as next-generation-sequencing) will be used to identify expressed genes while simultaneously quantifying differences in expression levels in select samples. This will be followed by Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) to verify differential expression and examine expression in additional time points and treatments.

For the initial libraries, RNA will be pooled (n=6) from oysters with naturally associated microbes to make 4 libraries; 1) ~380 pCO_2, week one, 2) 970 pCO_2, week one, 3) ~380 pCO_2, week four, 4) 970 pCO_2, week four. The four mRNA-seq libraries will be constructed and 680 Mb of sequencing (1 lane) will be completed for each library on an Illumina GA II (mRNA-Seq Sample Prep and Cluster Generation Kits; Illumina). Library construction and sequencing will be carried out by the High-Throughput Genomics Unit (HTGU), Department of Genome Sciences, University of Washington. This effort will generate ~ 25 million, 36 bp sequencing reads (based on Roberts, unpubl. data). The PI has experience incorporating this approach into several projects with 6 different aquatic species including the hard clam (Roberts, unpublished data). While it is difficult to predict results of expression data across conditions, based on similar efforts at least 750, ~400 bp unique consensus sequences will be generated that will be annotated using sequence similarity algorithms (BLAST). These consensus sequences will then be used as a reference to carry out differential

expression analysis for each of the four libraries (Genomics Workbench – CLC-Bio). While it is difficult to predict the number of differentially expressed products, a similar effort with trout using only two samples (two lanes) revealed over 100 differentially expressed genes (Roberts, unpublished).

To verify differential expression in the four samples used to make libraries, qRT-PCR will be carried out as previously described (Roberts et al 2009). Briefly, RNA will be reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen), which includes a DNAse step to eliminate genomic DNA carry-over. qRT-PCR reactions will contain the following: 1.0uL cDNA, 0.04uM forward/reverse primers, Brilliant SYBR GreenQPCR Master Mix (Stratagene). An Opticon 2 thermocycler (Bio-Rad) will be used to quantify gene expression. Analysis of PCR data will be carried out based on the kinetics of individual PCR reactions using Real-time PCR Miner v2.1 (Zhao and Fernald, 2005). All qPCR data will be normalized to a corresponding reference gene (18s ribosomal) and expressed as fold increase over minimum. A minimum of eight genes will be selected for qRT-PCR analysis. qRT-PCR will also be carried out in oyster samples from other pCO_2 treatments and all time points. The reason qRT-PCR will be used is that it would be cost prohibitive to construct sequencing libraries for all samples. A primary reason this short-read sequencing platform is being used is that this approach lacks any bias that using a pre-made microarray might possess, is a cost-effective approach for gene discovery, and simultaneous expression analysis.

Microbial Community Structure
We will assess the impact of pCO_2 on the microbial community in the seawater associated with each pCO_2 treatment using an established rapid, high-throughput fingerprinting method. In particular, we will use Automated Ribosomal Intergenic Spacer Analysis (ARISA) to create a genetic fingerprint of the microbial community in each sample. ARISA uses the ribosomal RNA internally transcribed spacer (ITS), a region of DNA known to vary in size in different microbial species, to create a genetic fingerprint of the diverse microbial community in each sample (Fisher & Triplett 1999).

ARISA analyses will be conducted on gill tissue of oysters to examine the role the host has on community structure. DNA will be extracted using standard methods (Qiagen Kit). 0.18 - 0.22 grams of oyster tissue will be homogenized with a Tissue Tearor (BioSpec Products, Inc.). DNA extraction will be performed using the QIAamp DNA Stool mini kit (Qiagen) following the protocol for stool pathogen detection provided by the manufacturer. Extracted DNA will be diluted to 1 - 5 ng/uL DNA and amplified by PCR, targeting the bacterial rRNA gene using standard methods. Samples for

microbial community analysis will be taken at the start of the experiment (t=0) and at the end of each week.

The ITS will be amplified using universal bacterial primers (16S-1392F and 23S-R, Brown *et al.* 2005), including a fluorescently labeled forward primer. For each sample, four independent PCRs will be performed, pooled, ethanol precipitated to remove unincorporated primers and run on a MegaBace 96 capillary sequencer along with ROX labeled size standards (50-1500 bp ladder, BioVentures, Inc). This sequencer is routinely used for fragment analysis and can resolve differences of 2 bp in fragments in the 300-400 bp range and differences of 10 bp for larger fragments (1000-1500 bp). Fragment lengths will be sized using DAX software (http://www.dax.nl/dax.htm) using a signal to noise cutoff to verify presence of peaks. We will also use up to date binning methods for assigning peak sizes to account for methodological variation (Hewson & Fuhrman 2006).

The impact of pCO_2 and oyster presence on microbial community composition will be assessed using an Analysis of Similarity (ANOSIM; Clarke and Warwick 2001). NMDS will be used to visualize differences in microbial community composition among samples. We will also use NMDS and ANOSIM to test for a relationship between pH (pCO_2) treatment and microbial community composition both for microbes from natural oyster microbial communities and from oysters that have been treated and cultured in autoclaved seawater. We will examine the relationship between the microbial community found within oyster tissue and that in the surrounding water in each experimental unit using a Mantel test. This will offer insight into the degree to which these two communities are independent and whether or not the oyster tissue conditions select for particular taxa from the pool available in the water. We will also assess the relationship between measures of oyster physiological response (see above) and microbial community composition using similar approaches.

Indirect Biological Effect / Secondary Stressor

To address the third objective, at the end of one month, the remaining oysters in all systems and treatments will be subjected to a pathogen challenge (details in Sampling section above). Days to mortality will be assessed to determine the indirect effect of increased pCO_2 on overall oyster health. These results will provide insight more broadly on the magnitude of the impact of elevated pCO_2 the ability of oysters to deal with a secondary stressor. Adding a secondary stressor may illuminate underlying condition that is not readily apparent, but leaves the oyster more vulnerable to pathogen infection.

To determine whether changes in overall microbial community composition are associated with changes in pathogen load and time to mortality for oysters, ARISA will be carried out as described and levels of pathogen will be determined by qPCR. Briefly, DNA will be extracted from individual gill samples using the Qiagen DNA stool kit, according to the manufacturer's protocol. qPCR will be carried out similarly as to the description above for gene expression analysis, except that genomic DNA will be used as opposed to cDNA. Primers used to quantify *V. tubiashii* levels will be either those described by Lee et al 2002 or Marushchak et al. 2008. A multivariate approach such as CCA and TWINSPAN will be used to assess the relationship among bacterial community composition in the oyster tissue, time to mortality and pathogen load.

Expected Outcomes and Impacts

The predicted increases in atmospheric CO_2 levels will lead to a drop in oceanic pH between 0.3-0.5 units by the year 2100 (Zeebe and Wolf-Gladrow 2001, Caldeira and Wickett 2005). The potential impact of this decrease in oceanic pH on marine organisms is not completely understood. Recent research has focused primarily on the negative effects that ocean acidification may have on limited processes in marine invertebrates. Very little research has incorporated microbial communities into the comprehensive analysis of ocean acidification impacts. Due to the complexity of ocean ecosystems and the central role of microbial communities to maintaining their health and function, an integrated approach to investigating the effects of ocean acidification will be necessary to accurately anticipate the impacts of CO_2-induced decreases in oceanic pH on marine organisms.

Our proposal aims to address this issue by asking three questions. The questions and associated expectations in the form of hypotheses are as follows:

1) How will elevated pCO_2 directly impact oyster physiology?

H) Elevated pCO_2 will induce a suite of early response genes that will decrease in expression over time.

H) Elevated pCO_2 will impact normal physiological processes due to the role of pH in general enzyme activity and homeostasis maintenance.

2) How will elevated pCO_2 impact microbial community structure?

H) Elevated pCO_2 will directly alter planktonic microbial community composition as optimal environmental conditions vary across taxa.

H) Elevated pCO_2 will be associated with changes in microbial community structure/composition within the oyster host in response to changes in host biology.

3) What are the indirect effects of ocean acidification on oysters?

H) Elevated pCO_2 will negatively impact the oyster immune system such that when subsequently faced with a pathogen challenge, oyster mortality will increase.

H) Microbial community composition will be associated with changes in pathogen load and time to mortality for oysters faced with a pathogen challenge.

The molecular approaches proposed as part of this study will allow us to examine parameters that go beyond basic effects such as growth rate and mortality. For instance, by examining differential gene expression in oysters under three CO_2 treatments, we will be able to identify interactions with other biological process by identifying physiological processes impacted by decreased pH. In addition, ARISA offers an effective method for assessing changes in the overall microbial community composition, thus providing insight into the potential role that the community plays in the oyster response as well as in the dynamics of pathogens. This type of research integration is critical in predicting real-world scenarios of CO_2-driven climate change.

The information gained through this research will be of immediate value to other researchers. The techniques described (qPCR, ARISA) are readily available at most universities and research institutions. Currently nothing is known regarding the global transcriptomic response of adult oysters to elevated CO_2 nor how microbial community composition responds and influences processes in the scenarios examined here. Examples of products include identification of genes that are regulated that could be further characterized and underlying mechanisms explored. In addition, others could examine microbial shifts in greater taxonomic detail using our data as a foundation for experimental design. Along the same lines approaches and results of this project could be applied to other organisms and larger and longer-term mesocosms.

The results of this research will also be valuable to resource managers and aquaculturists. While the project will not have the ability to alleviate ocean acidification, it will provide information on how changes in microbial flora, as well as acute and long term decreases in pH, might affect production, particularly in response to pathogen outbreaks. Even before we realize decreased pH as a result of large scale processes, local acute changes will (and have) occur related to local biological processes (ie photosynthesis, H_2S production, upwelling). This issue has become particularly relevant on the West Coast, where highly publicized shellfish mortality events have been associated with low pH events. Our study will provide additional insight on

how the interactions between oysters, microbial communities and pH can affect an oyster's response to the secondary stressor of pathogen exposure. This information will be useful to the aquaculture industry to identify, gauge, and respond to potential catastrophic conditions. See attached Letter of Support from Rick Karney; Director – Martha's Vineyard Shellfish Group, Inc.

Finally, the public will receive great benefit as well. The shellfish industry is a renewable resource that contributes greatly to the local and national economies. Oysters and other shellfish are also a popular food source throughout the world. The knowledge gained with this work will help to emphasize that the current output of anthropogenic CO_2 in our atmosphere will have a noticeable effect upon marine ecosystems that will, in turn, impact human quality of life. This information will provide people with a more concrete concept of how elevated atmospheric CO_2 will impact their lives.

Evaluation of Project Outcomes and Outreach

Completion of the proposed research will provide fundamental information on physiological and community responses to decreased pH in integral components of coastal ecosystems - shellfish and microbes. In addition, we will gain an understanding of the indirect biological impacts of ocean acidification by addressing the role of pH and microbial community on a disease challenge event. Results of this research will be made available to other scientists, natural resource managers and policy makers, however it is essential that the general public be made aware of these findings. Thus, we see this project directly in line with the goals of the Sea Grant program. Woods Hole Sea Grant excels in presenting the results of fundamental research to the general public. Given that ocean acidification is a direct result of human contribution to global CO_2 levels, simply informing the public on the rationale behind the research will lead to increased efforts by people to reduce their carbon footprint. Conducting the experiment at the Marine Biological Laboratory will also increase awareness of the bigger issue, as well as the research results, given the large number of public tours.

Several mechanisms will be used to disseminate the results of our research in a timely manner including publications, presentations, and institutional websites. We expect at least two peer-reviewed publications from this research and the work will be the primary thesis material for the graduate student funded as part of this project. Results of this work will be presented at several scientific meetings such as the National Shellfisheries Association Conference, the American Society of Microbiology or the Coastal and Estuarine Research Federation. In addition, during the summer of 2010, Roberts will be speaking at the Gordon Conference on Oceans and Human Health at the University of New England (tinyurl.com/ocean2010) where

preliminary results and the innovative use of transcriptomics will be presented. The fact the home institution of the PIs is in Washington will also provide a valuable mechanism for sharing the data with a larger audience. This research will be integrated into the courses the PIs teach at the University of Washington including; FISH310: Biology of Shellfishes, FISH441 Environmental Physiology, and FISH507 Bioinformatics. Horner-Devine will also integrate results as a case study in her introductory biology class (BIO180), thus reaching approximately 600 undergraduate students. Horner-Devine will also develop a unit of focusing on marine microbes highlighting this local work for her honors course in microbial ecology and evolution for 25 non-science majors (HAS221).

The lab of the co-PI is also actively involved in open science practices with all personnel keeping open access electronic notebooks (genefish.wikispaces.com) that are publicly accessible. Through these web-based, open access methods of sharing data, the public can follow the efforts and results of this research in near-real time throughout the duration of the project.

Project Management

Overall project management will be the responsibility of Roberts. As part of the development of this proposal, discussions have already occurred with the Director of the Marine Resources Center at the Marine Biological Laboratory (Scott Lindell; see letter of support) to insure this project can be effectively carried out at the facility. Roberts will also be responsible for assessing transcriptomic response in oysters including coordinating sequencing with the High-Throughput Genomics Unit. Horner-Devine will be responsible for microbial community analysis. Roberts and Horner-Devine will co-advise a graduate student whose thesis will be based on the research proposed here.