OMB Control No. 0648-0362
Expiration Date 3/31/2008

SEA GRANT PROJECT SUMMARY FORM

(1) INSTITUTION: Woods Hole Oceanographic Instituion (1a) ICODE:
(2) TITLE: Characterizing the Ecology of *Roseovarius crassostreae*, the Causative Agent of Juvenile Oyster Disease
(3) PROJECT NUMBER: R/B-177
(4) REVISION DATE:
(5) PROJECT STATUS: New
(6) INITIATION DATE: 6/1/07
(7) COMPLETION DATE: 5/31/08
(8) SUB PROGRAM:

(9) PRINCIPAL INVESTIGATOR: McDowell, Judith E. (9a) EFFORT: N/C
(9b) AFFILIATION: Woods Hole Oceanographic Institution
(9c) AFFILIATION CODE:

(10) CO-PRINCIPAL INVESTIGATOR:
(10a) EFFORT:
(10b) AFFILIATION:
(10c) AFFILIATION CODE:

(11) ASSOCIATE INVESTIGATOR 1:
(11a) EFFORT:
(11b) AFFILIATION:
(11c) AFFILIATION CODE:

(12) ASSOCIATE INVESTIGATOR 2:
(12a) EFFORT:
(12b) AFFILIATION:
(12c) AFFILIATION CODE:

(13) S.G. FUNDS: $96,890
(14) MATCHING FUNDS: $53,810
(15) LAST YEAR'S SG FUNDS:
(16) LAST YEAR'S MATCHING FUNDS:
(17) PASS-THROUGH FUNDS:
(18) LAST YEAR'S PASS-THROUGH FUNDS:
(19) RELATED PROJECTS:
(20) PARENT PROJECTS:
(21) SEA GRANT STRATEGIC PLAN CLASSIFICATION:
II. Coastal Ecosystem Health and Public Safety, A. Coastal Ecosystem Health,
92 - Biological Contaminants

(22) OBJECTIVES:
In order to better understand the ecology of *R. crassostreae* and factors influencing disease, we are proposing the following three research objectives;

> *1) Use real-time PCR to characterize* R. crassostreae *distribution and abundance in and near oyster aquaculture sites and correlate with environmental conditions.*
>
> *2) Assess physiological differences in planktonic and attached lifestyles of* R. crassostreae *by varying culture conditions and by evaluating changes in bacteria from infected oysters.*
>
> *3) Communicate with the oyster industry and all interested parties the details of our findings and techniques used.*

(23) METHODOLOGY:

A primary component of the proposed research is to characterize *R. crassostreae* distribution and abundance near oyster aquaculture sites. This will be accomplished by sampling three oyster production sites in Massachusetts, Connecticut, and New York. Samples for analysis will be taken from the inner shell surfaces and mantle edges of oysters during routine sampling events. One liter of the seawater in oyster holding containers will be collected and filtered to concentrate potential *R. crassostrea.* Bacterial films on the side of the containers in which the oysters are suspended will also be sampled by swabbing the sides of the container.

All samples will be analyzed using a real-time quantitative PCR assay. Specifically, dual-labeled probe (e.g. FAM and BHQ1) molecular technology will be used, targeting the same DNA sequence used in the conventional PCR assay developed in the Boettcher lab (collaborator on current proposal). The assay will be validated in the laboratory using pure cultures of *R. crassostreae* and experimentally infected oysters.

Using quantitative PCR to examine *R. crassostreae* will provide valuable data regarding prevalence, however this approach will not provide information regarding how the lifestyle and/or growth phase of *R. crassostreae* may influence its ability to colonize oysters. In order to investigate this question, expressed genes (RNA) associated with the presence of a flagella or polar fimbriae will be examined. Flagella are most commonly used in planktonic lifestyles; fimbriae are alternately employed for attachment of bacterial cells to biotic and/or abiotic surfaces. A degenerative primer based approach will be used it to identify select genes and expression patterns will be confirmed using quantitative reverse-transcription-PCR.

Providing current information regarding the progress of our research will be another objective and will be carried out in a variety a means including; extension agents, web sites, and local SeaGrant offices. All findings will be published in referreed journal articles. Investigators will make presentations at local shellfish officers meeting, local aquaculture organization meeting, regional meetings, and national aquaculture meetings. Any evaluation methods for detection of pathogenic *R. crassostreae* will be provided to other diagnostic laboratories through direct communications and written procedures.

(24) RATIONALE:

Juvenile oyster disease (JOD) can cause significant mortalities among commercially reared Eastern oysters*, Crassostrea virginica*, on the northern Atlantic coast of the United States. Mortality rates have been reported to reach greater than 90% of total production at JOD-enzootic sites in Maine, Massachusetts and New York. Of particular concern is the sudden appearance of JOD in previously unaffected areas. Little is known about the processes that influence the distribution and abundance of the causative agent, the α-proteobacterium *Roseovarius crassostreae*. Similarly, the factors involved in the initial stages of host recognition by the bacteria and subsequent colonization that results in disease remain to be elucidated.

Using state of the art molecular tools and collaborating with oyster growers throughout the region, we will provide information how environmental conditions affect *R. crassostreae* prevalence, host recognition, and colonization. These data will be of immediate benefit for risk assessment, site selection and developing general management practices. The production of a sensitive, accurate, specific and field tested assay for *R. crassostreae* will provide researchers an invaluable tool to examine JOD.

While management strategies are helping to minimize JOD outbreaks, the annual impact of the disease is unpredictable, and production is limited because of constraints on when seed can be safely deployed. JOD can also occur without warning in previously unaffected areas with disastrous results for growers unaccustomed to dealing with the disease. It is important to understand how JOD emerges in new areas, which in turn, requires understanding the ecology of the causative agent.

OMB Control No. 0648-0362

Expiration Date 3/31/2008

SEA GRANT PROJECT SUMMARY FORM

(1) INSTITUTION: Woods Hole Oceanographic Instituion (1a) ICODE:
(2) TITLE: Characterizing the Ecology of *Roseovarius crassostreae*, the Causative Agent of Juvenile Oyster Disease
(3) PROJECT NUMBER: R/B-177
(4) REVISION DATE:
(5) PROJECT STATUS: New
(6) INITIATION DATE: 6/1/08
(7) COMPLETION DATE: 5/31/09
(8) SUB PROGRAM:

(9) PRINCIPAL INVESTIGATOR: McDowell, Judith E. (9a) EFFORT: N/C
(9b) AFFILIATION: Woods Hole Oceanographic Institution
(9c) AFFILIATION CODE:

(10) CO-PRINCIPAL INVESTIGATOR:
(10a) EFFORT:
(10b) AFFILIATION:
(10c) AFFILIATION CODE:

(11) ASSOCIATE INVESTIGATOR 1:
(11a) EFFORT:
(11b) AFFILIATION:
(11c) AFFILIATION CODE:

(12) ASSOCIATE INVESTIGATOR 2:
(12a) EFFORT:
(12b) AFFILIATION:
(12c) AFFILIATION CODE:

(13) S.G. FUNDS: $97,036
(14) MATCHING FUNDS: $44,172
(15) LAST YEAR'S SG FUNDS: $96,890
(16)LAST YEAR'S MATCHING FUNDS: $53,810

(17) PASS-THROUGH FUNDS:
(18) LAST YEAR'S PASS-THROUGH FUNDS:
(19) RELATED PROJECTS:
(20) PARENT PROJECTS:
(21) SEA GRANT STRATEGIC PLAN CLASSIFICATION:
II. Coastal Ecosystem Health and Public Safety, A. Coastal Ecosystem Health,
92 - Biological Contaminants

(22) OBJECTIVES:
In order to better understand the ecology of *R. crassostreae* and factors influencing disease, we are proposing the following three research objectives;

> *1) Use real-time PCR to characterize* R. crassostreae *distribution and abundance in and near oyster aquaculture sites and correlate with environmental conditions.*
>
> *2) Assess physiological differences in planktonic and attached lifestyles of* R. crassostreae *by varying culture conditions and by evaluating changes in bacteria from infected oysters.*
>
> *3) Communicate with the oyster industry and all interested parties the details of our findings and techniques used.*

(23) METHODOLOGY:

A primary component of the proposed research is to characterize *R. crassostreae* distribution and abundance near oyster aquaculture sites. This will be accomplished by sampling three oyster production sites in Massachusetts, Connecticut, and New York. Samples for analysis will be taken from the inner shell surfaces and mantle edges of oysters during routine sampling events. One liter of the seawater in oyster holding containers will be collected and filtered to concentrate potential *R. crassostrea.* Bacterial films on the side of the containers in which the oysters are suspended will also be sampled by swabbing the sides of the container.

All samples will be analyzed using a real-time quantitative PCR assay. Specifically, dual-labeled probe (e.g. FAM and BHQ1) molecular technology will be used, targeting the same DNA sequence used in the conventional PCR assay developed in the Boettcher lab (collaborator on current proposal). The assay will be validated in the laboratory using pure cultures of *R. crassostreae* and experimentally infected oysters.

Using quantitative PCR to examine *R. crassostreae* will provide valuable data regarding prevalence, however this approach will not provide information regarding how the lifestyle and/or growth phase of *R. crassostreae* may influence its ability to colonize oysters. In order to investigate this question, expressed genes (RNA) associated with the presence of a flagella or polar fimbriae will be examined. Flagella are most commonly used in planktonic lifestyles; fimbriae are alternately employed for attachment of bacterial cells to biotic and/or abiotic surfaces. A degenerative primer based approach will be used it to identify select genes and expression patterns will be confirmed using quantitative reverse-transcription-PCR.

Providing current information regarding the progress of our research will be another objective and will be carried out in a variety a means including; extension agents, web sites, and local SeaGrant offices. All findings will be published in referreed journal articles. Investigators will make presentations at local shellfish officers meeting, local aquaculture organization meeting, regional meetings, and national aquaculture meetings. Any evaluation methods for detection of pathogenic *R. crassostreae* will be provided to other diagnostic laboratories through direct communications and written procedures.

(24) RATIONALE:

Juvenile oyster disease (JOD) can cause significant mortalities among commercially reared Eastern oysters*, Crassostrea virginica*, on the northern Atlantic coast of the United States. Mortality rates have been reported to reach greater than 90% of total production at JOD-enzootic sites in Maine, Massachusetts and New York. Of particular concern is the sudden appearance of JOD in previously unaffected areas. Little is known about the processes that influence the distribution and abundance of the causative agent, the α-proteobacterium *Roseovarius crassostreae*. Similarly, the factors involved in the initial stages of host recognition by the bacteria and subsequent colonization that results in disease remain to be elucidated.

Using state of the art molecular tools and collaborating with oyster growers throughout the region, we will provide information how environmental conditions affect *R. crassostreae* prevalence, host recognition, and colonization. These data will be of immediate benefit for risk assessment, site selection and developing general management practices. The production of a sensitive, accurate, specific and field tested assay for *R. crassostreae* will provide researchers an invaluable tool to examine JOD.

While management strategies are helping to minimize JOD outbreaks, the annual impact of the disease is unpredictable, and production is limited because of constraints on when seed can be safely deployed. JOD can also occur without warning in previously unaffected areas with disastrous results for growers unaccustomed to dealing with the disease. It is important to understand how JOD emerges in new areas, which in turn, requires understanding the ecology of the causative agent.

PROJECT DESCRIPTION

INTRODUCTION

Oyster (*Crassostrea sp.)* populations in the United States are a valuable component of the environment, supporting important capture fisheries and an expanding aquaculture industry. On the East Coast, the eastern oyster (*Crassostrea virginica*) is the predominant shellfish species of interest. During the late 1990's there was a precipitous decline in aquaculture production of *C. virginica* (Figure 1. A) that corresponded to a decrease in revenue (Fig 1. B) (FAO, 2006). In the past few years there has been a steady increase in global aquaculture production (Figure 1), which unfortunately is not mirrored in the capture fisheries for this species (FAO, 2006).

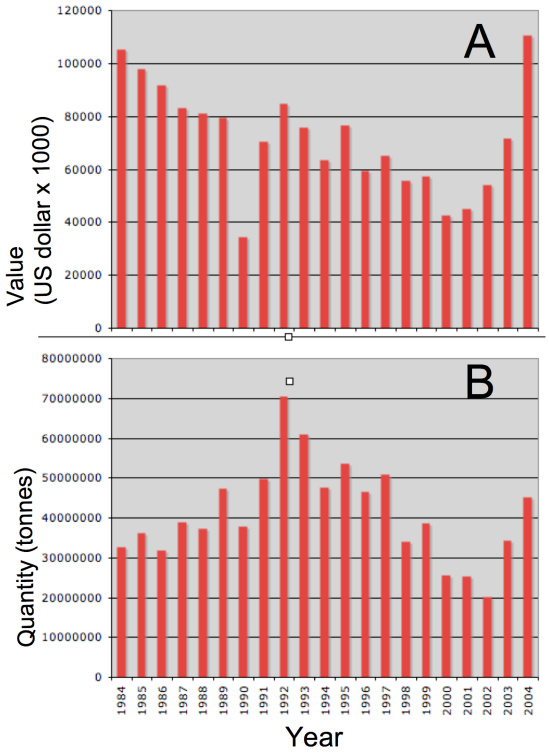


Figure 1. (A) Value of global aquaculture production of *Crassostrea virginca*. (B) Quantity (t) of aquacutlure produced *Crassostrea virginca*.

While there has been a slight increase in global production quantities of *C. virginica* over the past couple of years, the United States' production has not fully recovered from the devastating declines experienced in last 50 years, primarily the result of disease. There clearly continues to be a need to have an increased understanding of the diseases that threaten native and aquacultured oysters. The disease Dermo has received significant attention, as it is responsible for considerable mortality. As oyster production efforts continue to expand there should be increasing concern for emerging oyster threats such as Juvenile Oyster Disease.

Juvenile oyster disease (JOD) can cause significant mortalities among commercially reared Eastern oysters, *Crassostrea virginica*, on the northern Atlantic coast of the United States. Mortality rates have been reported to exceed 90% of total production at JOD-enzootic sites in Maine, Massachusetts and New York. Of particular concern is the recent appearance of JOD in previously unaffected areas such as Martha's Vineyard in 2003 (Boettcher & Maloy 2004). During September 2006, Sargents Cove LLC (Norwalk, CT) experienced significant mortality in seed (3-10mm) associated with JOD (Roberts and Denham, unpublished).

The disease is characterized by an abrupt reduction in growth, followed by the development of fragile and uneven shell margins, watery and emaciated tissues, and the deposition of abnormal proteinaceous material (conchiolin) on the inner shell surfaces by the inflamed shell epithelium. However conchiolin deposits are not always present, as the deposits were not observed in recent cases of JOD at Sargents Cove's hatchery and as reported by Boettcher and Ford (2006). Grossly observable lesions also tend to occur

2-3 weeks after the disease begins (Smolowitz, Boettcher personnal observation) and so are usually not seen till the disease is already causing mortalities. This was the case during the 2003 outbreak of JOD on Martha's Vineyard when shell changes were not detected until 2.5 weeks after Smolowitz diagnosed the mantle lesions using histopathological techniques. Juvenile oyster disease usually has the greatest impact on oysters between 6 and 30 mm shell height. High mortalities usually occur during the summer months when water temperatures exceed 20°C. While there is no known treatment for JOD, measures such as early deployment, use of selected stocks, and diligent husbandry have helped to minimize losses (Barber et al 1996, Barber et al. 1998, Lewis 2001, Ford & Borrero, 2001). There are also anecdotal accounts that changes in environmental conditions, such as salinity, can hinder the development of JOD (Boettcher & Maloy, unpublished).

The causative agent of JOD is an α-proteobacterium recently described as the novel species *Roseovarius crassostreae* (Boettcher et al. 2005; Boettcher et al. 2006). The bacterium is consistently recovered as the dominant species from oysters taken from JOD outbreak sites. The color of the bacteria colonies is usually beige to pinkish beige, however there are exceptions as was observed during the 2003 outbreak in Martha's Vineyard when the colonies were greenish yellow. The cells are Gram-negative, aerobic, rod or ovoid in appearance (Figure 2), and (as they require sodium for growth) are strictly marine. Phylogenetic analyses based on DNA sequencing information assigns *R. crassostrea* to the *Roseobacter* clade of the α-*Proteobacteria.* However, *R. crassostreae* is genetically, physiologically, and morphologically dissimilar to the other members of the *Roseovarius* genus (i.e. *R. tolerans* and *R. nubinhibens)* (Boettcher et al 2005).

Unfortunately, little is known about the processes that influence the distribution and abundance of *R. crassostreae* in the environment. Similarly, the factors involved in the initial stages of host recognition by the bacteria and subsequent colonization that results in disease remain to be elucidated. There are however, some clues. Work by Dang and Lovell (2000) has shown that alpha Roseobacteria are an important component of the bacterial film that develop in high numbers on surfaces exposed to sea water (potentially such items as bags, upwellers, etc). Even more interestingly, flagellar motility is clearly evident in wet mounts prepared from laboratory cultured *R. crassostreae* colonies. However, when examined by electron microscopy, a small percentage of cells in such preparations are observed to lack flagella. In their place are tufts of polar fimbriae consisting of dozens of protein fibrils (Boettcher et al. 2005). While flagella are most commonly used in planktonic lifestyles, fimbriae are

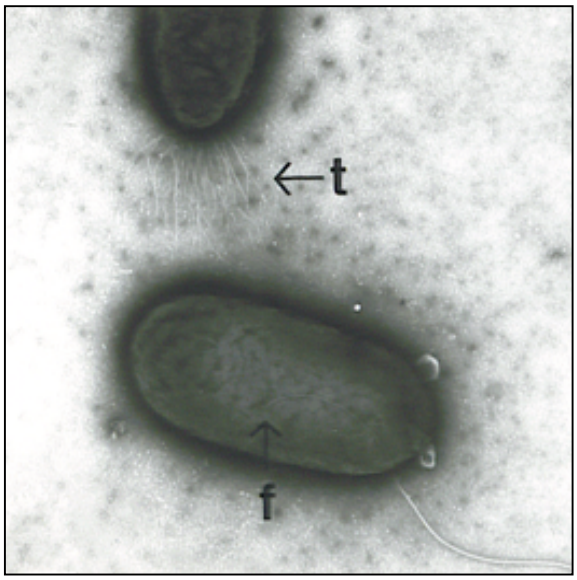


Figure 2. Electron micrograph of *Roseovarius crassostrea* from Boettcher *et al* 2005. Two cells; one with flagellum (f) and one displaying a polar fimbriae tuft (t).

alternately employed for attachment of bacterial cells to biotic and/or abiotic surfaces. In those cases where fimbriae are used to colonize a particular host, they are considered virulence factors. Examination of the inner shell surfaces of JOD-affected oysters has revealed dense microcolonies of bacteria (believed to be *R. crassostreae*) that are attached via their cells poles (Figure 3) (Boardman 2005). Thus, there is good evidence that these polar fimbriae are required for the colonization of oysters by *R. crassostreae.* Additionally, the occurrence of fimbriae is known to be one of the more important characteristics of increased virulence in *Escherichia* coli, a bacteria that is commonly found in all mammals intestines. The occurrence of fimbria in *E. coli* promotes colonization and subsequently destruction of the epithelium of the intestine and urinary bladder in mammals (Susa et al., 1996; Boyd and Hartl., 1998).

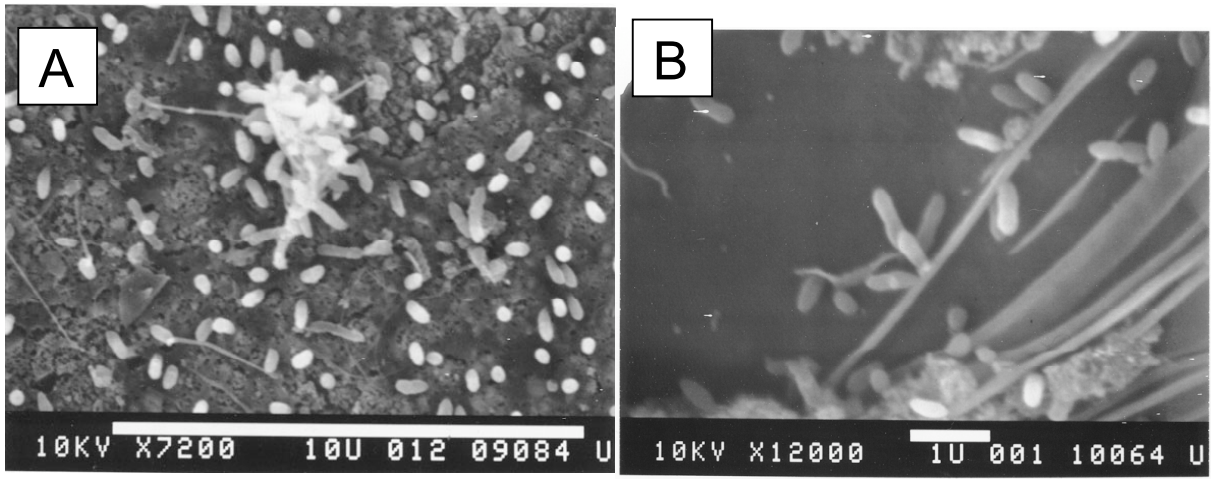


Figure 3. SEM images of JOD-affected oysters showing (A) bacterial cells attached at cell poles to the shell surface, (B) bacterial cells attached via their cell poles to conchiolin deposits on the inner shell. (Boardman 2005).

Current methods of *R. crassostreae* detection include conventional bacteriology, agglutination assays, and a standard polymerase chain reaction (PCR) based technique. The PCR assay is designed to amplify the internal transcribed spaces (ITS) region of *R. crassostreae* and effectively detects the presence of *R. crassostreae* directly from infected oysters without cross-reaction with other known species (Maloy et al 2005). This assay will be the basis for the development of a quantitative real-time PCR assay for *R. crassostreae*, a component of the current proposal. Quantitative real-time PCR involves coupling fluorescent detection with standard PCR amplification and has been used for disease detection in several commercially important marine organisms (e.g., Roberts and Goetz, 2004). In real-time, fluorescent emissions are recorded at each PCR cycle which allows accurate quantitation in the exponential phase of the PCR reaction. Real-time PCR has the ability to detect as little as one target cell and the dynamic range of real-time PCR is up to 10^7 – fold (compared to approximately 1000-fold in standard PCR). Recently this technology has been used to demonstrate that the water column is a reservoir for shellfish pathogens, human pathogens and other microorganisms (Lyons et al. 2006, Lyons et al. 2007, Bogan and Roberts unpublished)

RESEARCH PLAN

In order to better understand the ecology of *R. crassostreae* and factors influencing disease, we are proposing the following three research objectives;

1) Use real-time PCR to characterize R. crassostreae *distribution and abundance in and near oyster aquaculture sites and correlate with environmental conditions*

2) Assess physiological differences in planktonic and attached lifestyles of R. crassostreae *by varying culture conditions and by evaluating changes in bacteria from infected oysters.*

3) Communicate with the oyster industry and all interested parties the details of our findings and techniques used.

In order to complete the first objective, the ITS region used in standard PCR detection will be used to design (Primer Express, Applied Biosystems) two primers and an oligonucleotide probe containing a fluorescent dye (Figure 4). This assay will be as effective and accurate as the current detection method (Maloy et al 2005), however we will be able to increase throughput, streamline the protocol, and most importantly, accurately quantitate *R. crassostreae* levels. The real-time PCR method will be applied in the laboratory and in the field to demonstrate its effectiveness in diagnosis, testing control methods, and characterizing the population dynamics of *R. crassostreae*.

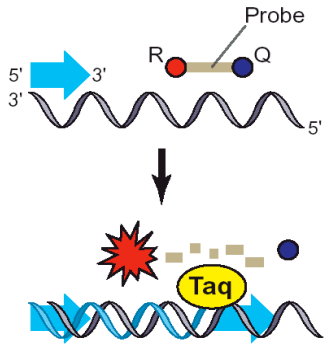


Figure 4: Schematic of probe function in Real-time PCR.

Using quantitative PCR to examine *R. crassostreae* will provide valuable data regarding prevalence, however this approach will not provide information regarding how the lifestyle and/or growth phase of *R. crassostreae* may influence its ability to colonize oysters. In order to investigate this question, expressed genes (RNA) will be isolated, as opposed to genomic DNA (i.e. ITS region). Whereas genomic DNA would be present in all life phases of *R. crassostreae*, there will be a suite of expressed genes that are specifically associated with the presence of a flagella or polar fimbriae (described above). By targeting one or more of these expressed genes in molecular quantitation assays, we can begin to learn more about the factors involved in the initial stages of host recognition and colonization. The identification of markers associated with specific life stages of *R. crassostreae* will be a valuable tool for studying the disease.

Specific Experimental Design

Laboratory Validation of Quantitative Assay – Real-time PCR

Initial testing of the real-time PCR assay will be performed with pure cultures of *R. crassostreae*. In all cases DNA will be extracted by standard methods (i.e. Qiagen DNeasy kits) and the assays performed on an Opticon2 Continuous Fluorescent Detection System. Assaying pure cultures will provide us with information regarding the detection levels, dynamic range, and allow parameter optimization. Oysters will then be experimentally exposed to *R. crassostreae* and sampled for increases in *R. crassostreae*. *R. crassostreae* bacteria to be used in this work are from a strain originally isolated by Boettcher from diseased oysters in Maine. Bacteria will be grown up in Sea Water Agar (Boettcher, personal communication) from -80º C maintained frozen cultures. Susceptible oysters (15-25mm) will be provided by the Martha's Vineyard Shellfish Group.

Since the real-time assay is based on a current test that has been validated (Maloy et al 2005) limited time and resources would be required for its development. Based on this prior research. Maloy et al. (2005) determined positions 109-126bp and 955-974bp of *R. crassostrea* CV919-312 (GenBank Accession # AF114484) had enough sequence dissimilarity with similar taxa to provide accurate targets for a specific diagnostic assay. For the quantitative assay, Primer Express Software (Applied Biosystems) will be used to design a set of two primers and an oligonucleotide probe containing a fluorescent reporter dye (FAM) at the 5'-terminus and a quencher (BHQ1) at the 3' terminus. Two combinations of probe / primers will be initially designed with the probe construction targeting the two sequence spans identified by Maloy et. al (2005), 109-126bp and 955-974bp. These two independent qPCR targets will initially be tested with only one selected for use. The assay conditions will be comprised of a two-step PCR cycling program (40 cycles) including denaturating at 94°C for 15 sec and annealing/extension at 60°C for 1 min. In real-time, data will be recorded as fluorescence emitted during the PCR reaction as an indicator of amplicon production. An example of the resulting data can be seen in Figure 5, a serial dilution series of nodavirus, a disease agent of fish the Roberts lab recently developed and real-time quantitative PCR assay for.

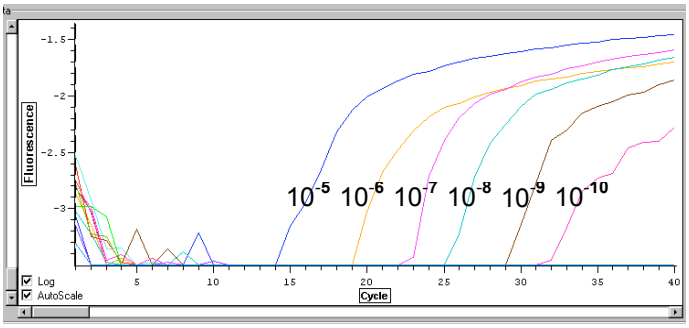


Figure 5. Fluorescence emitted during PCR reaction (indicating nodovirus detection) of serially diluted cod eye RNA.

Laboratory Validation of Quantitative Assay – Quarantine Facility and Oyster Trials

Roseovarius crassostreae culture and oyster exposure experiments will be conducted using methods to prevent transfer of the organism or infected oysters. Infection experiments will be conducted in plastic, isolated, covered 1 sq. ft. chambers that will sit in a fresh water bath of the appropriate temperature maintained using Teclima heater/chiller units. This system is in working order in the Smolowitz laboratory and has been used for similar temperature based experiments in the study of

protistian pathogens of clams. All information to date suggests that *R. crassostreae* is not a pathogen of fresh water animals, mammals or even of other marine animals. However, all infected oysters will be frozen and incinerated at the end of experiments. All cultures of *R. crassostreae* will be autoclaved before disposal. Gloves, paper, etc will be disposed using standard method (incinerated medical waste). Seawater used in exposure tanks and any metal items will be treated with bleach before disposal or cleaning. No potentially infected tissue, formites or cultures will be allowed near sea water systems at any time.

Oysters will be experimentally exposed to *R. crassostreae* and sampled for increases in the bacteria. Susceptible oysters (15-25mm) will be provided by the Martha's Vineyard Shellfish Group. These experiments will be carried out at two different temperatures (18°C, 22°C) and at two different oyster stocking densities; low=2000/meter and high=5000/meter. Oysters will be randomly divided into 8 groups (with replicates of two in each group) and exposed to *R. crassostreae* by water column exposure for 72 hours at a concentration of at least 10^6 cells/ml in the aquaria based on previous trials conducted by Boettcher. In seawater, an O.D. of 1.0 is approximately 5 x 10^{e8} cells/ml. Controls will not be exposed to *R. crassotreae* but otherwise handled at the treated animals. The water will be replaced after 72 hours and every 6 days afterwards to prevent severe fouling.

Animals will be fed once per day with fresh cultured algae (*Isochrysis* sp.) produced by the micro-algae production facility at the MBL (http://www.mbl.edu/aquaculture/algae/MAPF.html). Supplemental diet will be added as needed (e.g. Instant Algae , Reed Mariculture, Campbell, CA). Ten oysters will be removed from each container at 10 days and 30 days after exposure, or when mortality and/or gross lesions appear. The shell will be sterilely cracked open at the hinge, and material from the inner shell surfaces and mantle edges will be cultured on Sea Water Tryptone culture media at room temperature (using methods as described by K. Boettcher). These methods have already been in use in Smolowitz's laboratory for two years. Samples will be collected for real time qPCR, as described above, by removal and processing of a piece of mantle. The molecular assay will then be able to provide an average number bacteria/gram of tissue.

The oysters will also be evaluated grossly and processed for histological examination. After fixation in 10% formalin in seawater, the animals will be decalcified and processed using standard paraffin embedding methods (Howard et al., 2004). Two 6 µm step-sections containing mantle and overlying decalcified shell as well as other internal organs will be cut from each whole, decalcified oyster and stained with hematoxylin and eosin (MASS Histology Service, Worcester, MA). Stained tissue sections will be evaluated by Smolowitz using a Zeiss Axioskop 2. Lesions of the mantle edges and changes in the associated shell, as well as abnormalities in internal organs, will be evaluated and described. This data will be correlated with the real time qPCR (described above) and molecular evaluation for fimbriae (described below) in cultures taken during sampling of the inner shell and mantle edge.

This design will not only help develop our detection assay but will provide initial information on how environmental and density factors influence population levels of *R. crassostreae*, development of the potentially pathogenic fimbriated form and how these levels correlate with typical JOD lesions and resultant oyster mortality.

Field Sampling

A primary component of the proposed research is to characterize *R. crassostreae* distribution and abundance near oyster aquaculture sites. Multiple sites within the known range of *R. crassostreae* have been selected for sampling. Characteristics of all sampling sites are shown in Table 1.

Table 1. Sampling sites to be evaluated for *R. crassostreae*

Sampling Site	State	Investigator	JOD History
Katama Bay, Martha's Vineyard	MA	Karney	Consistent Presence
Sargents Cove	CT	Denham	Detected 2006
Frank M. Flower and Sons	NY	Reylea	> 10 yrs

Intensive sampling of a container (upweller or bag) holding JOD susceptible sized oysters will take place at one site on Martha's Vineyard (a site likely to have an epizootic event based on annual experiences in the last few years) with R. Karney. In Connecticut, samples will be taken at Sargents Cove LLC where an outbreak occurred in 2006. At a site at Flowers Oyster Co, which has a greater than 10-year history of JOD associated infection and mortality, D. Reylea and Gregg Rivara will be responsible for the sampling. Environmental sampling will occur 2 times/month in May, June and July and once per month in August, September and October in year one of the grant in order to obtain information during all stages of disease progression. Samples for real time analysis will be taken from the inner shell surfaces and mantle edges of 10 oysters at each sample period. One liter of the seawater in the container holding the oysters will be collected and filtered to concentrate potential *R. crassostrea* in a water sample directly exposed to vulnerably sized oysters. Bacterial films on the side of the containers in which the oysters are suspended will also be sampled by swabbing the sides of the container. Additionally, at a buoy or other attached structure at least 25 ft away from the oyster culture sites, both a one liter water sample and a swab of the bacterial film on the buoy (or other designated structure) will be taken at every sample period in order to evaluate *R. crassostrea* populations at a distance from potentially infected oysters. All samples will be taken using sterile techniques with supplies provided by the Marine Biological Laboratory.

All samples will then be analyzed using the real-time PCR assay. At each sampling site data loggers will be deployed to obtain comprehensive water parameters (i.e. temperature, salinity, flow) so that trends in *R. crassostreae* levels can be correlated with external factors. In addition, extension personnel (Rivara and Walton) will be in contact with hatcheries to identify specific requests for diagnosing JOD and will be able to ensure timely notification of potential JOD outbreaks. As samples are tested the results will immediately be made available to oyster farmers and the general public via direct contact and a website similar to a site used for disseminating information on Dermo research conducted by Roberts and Smolowitz; (http://www.mbl.edu/aquaculture/oyster/)

Identify markers for different life phases of R. crassostreae – Gene Identification

In order better understand host recognition and colonization mechanisms of *R. crassostreae*, we first need to identify markers that will allow us to distinguish planktonic and attached lifestyles of the bacteria. As described in the Introduction, flagella are most commonly used in planktonic lifestyles; fimbriae are alternately employed for attachment of bacterial cells to biotic and/or abiotic surfaces. Further, fimbriae appear to be involved in the attachment of *R. crassostreae* to the shell surfaces (and later the conchiolin deposits) of affected oysters. However, the molecular switch that controls the expression of flagella vs. fimbriae is not known. Expressed genes (or markers) specific to certain stages of *R. crassostreae* will be identified using a using a degenerative PCR primer protocol targeting candidate genes. This approach is becoming easier with the rapid rate of microbial genome sequencing. Notably, the genomes of five species in the *Roseobacter* clade are in the process of being annotated and several environmental sequencing efforts are underway. One example of genes that will be targeted includes one associated flagella formation (used in planktonic lifestyle). Currently there are ten *Roseovarius sp*. whole genome shotgun sequences in NCBI's GenBank with Flagella-associated genes identified (Genbank Accession numbers: NZ_AATQ01000027, NZ_AATQ01000013, NZ_AATQ01000002, AATQ01000002, AATQ01000013, AATQ01000027, NZ_AAMV01000010, NZ_AAMV01000005, AAMV01000005, AAMV01000010). Degenerative primers will be designed by aligning sequences with similar taxa to find regions of high homology. Figure 6 provides an example of an alignment of "flagellar biosynthesis protein B" in *Roseovarius sp*. (GenBank accession # NZ_AATQ01000027) and *Oceanicola sp*.

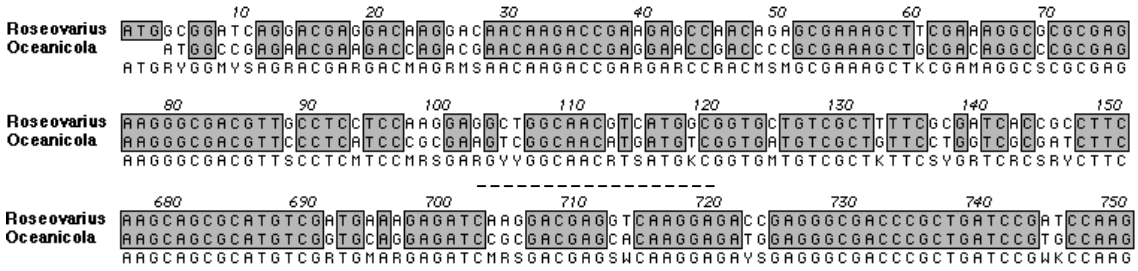


Figure 6. Nucleotide sequence alignment of flagellar biosynthesis protein B in *Roseovarius sp.* (Genbank accession # NZ_AATQ01000027) and *Oceanicola sp* as determined by BLAST analysis. Shaded regions indicated sequence identity and dashed line indicates break in consecutive sequence. Numbers correspond to bp in *Roseovarius* sequence.

Identification of genes associated with the presence of fimbriae will also be an important focus of our research efforts as fimbriae formation is linked to attachment to host organisms (eg. oysters). One example of a candidate gene that will be targeted is "fimbriae assembly related protein" ; *Acidiphilium sp*. GenBank # NZ_AAOO01000034. Using BLAST analysis, a homologous sequence was identified in a *Rhodobacter sp* (Gen Bank # CP000143; bp 3050033-3049434). Using the same technique outline in Figure 6, the *R. crassostrea* homologous gene will be identified

DNA sequencing will be carried to verify what gene product is being analyzed. Briefly, PCR products will be used to clone (TA TOPO 2.1, Invitrogen) large fragments of genes; and individual colonies will be randomly picked for plasmid preparations made using the RevPrep Orbit (GeneMachines). Plasmid preparations will be sequenced from the 5' end using the dideoxy chain termination method using Big Dye Terminator (Applied Biosystems) and the BK reverse vector primer. The reactions will precipitated and resuspended in Hi-Di Formamide with EDTA (Applied Biosystems) and run on an ABI Prism 3730 automated sequencer (Applied Biosystems). All new sequence information will be deposited in NCBI's GenBank and made available to other researchers.

Identify markers for different life phases of R. crassostreae – Expression pattern analysis

Quantitative PCR will then be carried out characterize level of expression. As opposed to using dual labeled probes as will be used for the other component of the proposal, SYBR green technology will be used. SYBR green technology is more similar to conventional PCR with only two primers (no probe) used in the PCR reaction, however a fluorescent dye that binds to double stranded DNA (PCR product).

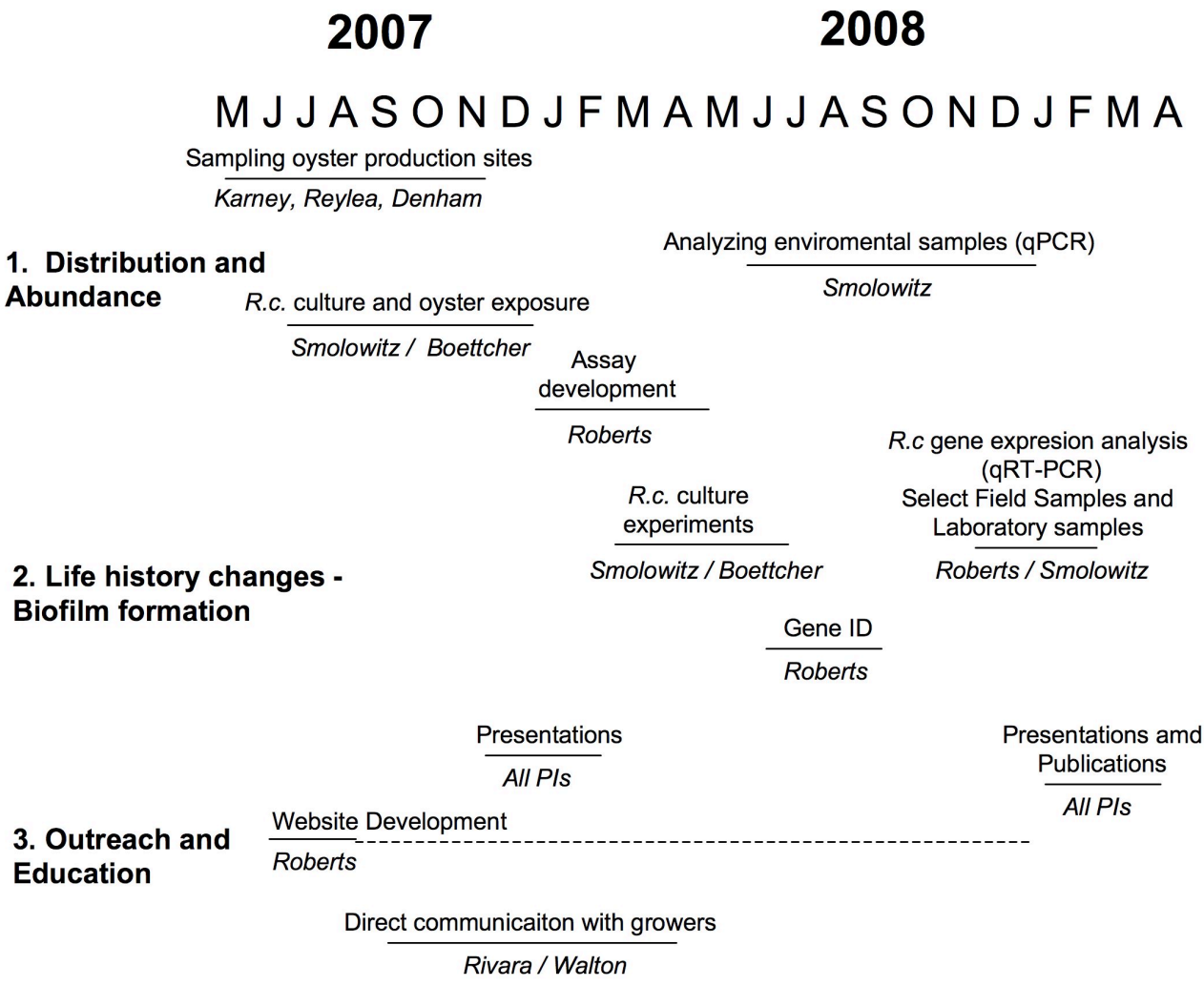
To identify changes in expression, total RNA extracted from *R. crassostreae* will be analyzed using real time RT-PCR (Brilliant SYBR Green QRT-PCR Master Mix Kit, 1-Step, Stratagene) in an Opticon Continuous Fluorescence Detection System (MJ Research). For all real time assays, melting curves will be analyzed to verify that no primer dimers are formed and that C_T values represent the desired amplicon. C_T values will then be converted to relative abundance levels based on their respective standard curves and normalized to the corresponding 18S RNA values.

Culture techniques developed by Boettcher will be essential in validating the markers. Log-phase cultures of *R. crassostreae* grown with shaking contain predominantly actively planktonic (flagellated) cells. In contrast, if allowed to settle, the cells will readily form biofilms on the surfaces of glass slides and polystyrene plates (Boettcher unpublished). In this study, *R. crassostreae* will be tested at various phases of growth, and under various conditions of temperature, pH, and salinity to determine those conditions most favorable for biofilm formation. Samples will be harvested at the planktonic and attached stages, RNA extracted, and gene expression patterns compared. During the second year of the proposed research, archived samples from field sampling will be processed to examine environmental effects on life phases of *R. crassostreae,* where *R. crassostrea* was detected using the dual-labeled probe qPCR assay.

Outreach and Education

While carrying out the proposed research we will monitor selected potential outbreaks of JOD outside of specific sampling sites (described below) with the assistance of extension agents participating on the proposal (Bill Walton, Aquaculture Specialist for Barnstable County, MA; Gregg Rivara, Cornell Cooperative Extension Agent, NY). Efforts will be make to incorporate limited requests for diagnostics into the current research plan, however it would be difficult to analyze a large number of samples given the potential resources needed. If the proposed research was funded this would significantly reduce any costs associated with diagnostic services. Information regarding the environmental conditions associated with JOD outbreaks (provided by growers and extension agents) will help in the interpretation of our laboratory experiments. Providing current information regarding the progress of our research will be a primary component of our activities and will be carried out in a variety a means including; extension agents, web sites, and local SeaGrant offices. All findings will be published in referreed journal articles. PIs will make presentations at local shellfish officers meeting, local aquaculture organization meeting (with the help of the extension agents), regional meetings (NACE) and national aquaculture meetings (NSA). Walton and Rivara will help the PIs to produce a fact sheet that can be provided to culturists and other extension agents in the northeast U.S. All information and updates on the research will be published on a website. Finally, any evaluation methods for detection of pathogenic *R. crassostreae* will be provided to other diagnostic laboratories through direct communications and written procedures.

TIMELINE


2007
2008
M J J A S O N D J F M A M J J A S O N D J F M A
Sampling oyster production sites
Karney, Reylea, Denham
1. Distribution and Abundance
Analyzing enviromental samples (qPCR)
Smolowitz
R.c. culture and oyster exposure
Smolowitz / Boettcher
Assay development
Roberts
R.c gene expresion analysis (qRT-PCR)
Select Field Samples and Laboratory samples
Roberts / Smolowitz
R.c. culture experiments
Smolowitz / Boettcher
2. Life history changes - Biofilm formation
Gene ID
Roberts
Presentations
All PIs
Presentations amd Publications
All PIs
3. Outreach and Education
Website Development
Roberts
Direct communicaiton with growers
Rivara / Walton

ROLE OF PROJECT PERSONNEL

Steven Roberts (Marine Biological Laboratory / University of Washington)
Roberts has extensive experience in molecular techniques, including gene expression analysis. He will be responsible for developing the real-time quantitative PCR assay to detect *R. crassostreae*. Related to objective two of the proposed research Roberts will be responsible for characterizing differences in gene expression between different life history stages of *R. crassostreae* using a degenerative primer based approach. Specifically, planktonic colonies will be compared to colonies that form a biofilm (e.g. possess flagella, and possible more virulent). Roberts also be responsible for maintaining web-based resources and presenting / coordinating project data.

Roxanna Smolowitz (Marine Biological Laboratory)
Smolowitz is a traditionally trained veterinary pathologist, has extensive experience studying bivalve disease. Smolowitz will be responsible for culturing *R. crassostrea*, including carrying out the oyster exposure trials in the first months of the proposed research design to validate the real-time quantitative assay. Also during year one, the Smolowitz lab will be responsible for delivery of sampling supplies to the three collaborators that will be responsible for field sampling at aquaculture production sites. Samples will return to the Smolowitz lab for archiving, processing (i.e. DNA extraction), and characterization via application of the real-time quantitative assay. Smolowitz will carryout *R. crassostrea* culture experiments to isolate bacteria in the planktonic stage and colonies predominantly forming biofilms. Following the identification of genes putatively indicative of specific life stages (Roberts) the Roberts lab and Smolowitz labs will work together to describe gene expression patterns using quantitative RT-PCR.

Letter of support attached.

Katherine Boettcher
Boettcher has extensive experience working with *R. crassostrea* and characterizing outbreaks of JOD. The current proposal is based on research results from her laboratory, including the PCR diagnostic and *R. crassostreae* culture techniques. Boettcher will act as a consultant on the current project assisting the Smolowitz lab with 1) initial bacteria culture and oyster exposure trials, and 2) *R. crassostreae* culture conditions designed to induce flagella biosynthesis / biofilm formation.

Letter of support attached.

Rick Karney (Martha's Vineyard Shellfish Group)
Karney will be responsible for providing oyster seed to the Marine Biological Laboratory for exposure trials during year one. He will also assist *Jack Blake* in routine field sampling of an oyster production site likely to have an epizootic event.

Letter of support attached.

Jack Blake (Sweet Neck Farm)
Blake, an oyster grower on Martha's Vineyard, will facilitate sampling of his oyster production site likely to have an epizootic event.

Letter of support attached.

Geoff Denham (Sargents Cove LLC)
Denham will be responsible for routine field sampling at an oyster production site in CT that has experienced JOD outbreaks (recent occurrence in Fall 06).

Letter of support attached.

Dave Reylea (Flower's Oyster Company)
Reylea will be responsible for routine field sampling at an oyster production site in NY that has a greater than 10-year history of JOD associated infection and mortality.

Letter of support attached.

Gregg Rivara (Cornell University Cooperative Extension of Suffolk County)
As an aquaculture specialist and extension agent, Rivara will provide information on the occurrences of JOD in the region and assist in disseminating results of our research to growers. Rivara will also assist Reylea in sampling at Flower's Oyster Company.

Letter of support attached.

Bill Walton (Barnstable County's Cape Cod Cooperative Extension &
Woods Hole Oceanographic Institution Sea Grant)
Walton will be responsible for providing information on JOD outbreaks in the Massachusetts region and assisting in disseminating results of our research to growers and the public.

Letter of support attached.

OUTPUT

Information on how environmental conditions affect *R. crassostreae* prevalence, host recognition, and colonization will be of immediate benefit for risk assessment, site selection and developing general management practices. The production of a sensitive, accurate, specific and field tested real-time assay for *R. crassostreae* will provide researchers and diagnosticians will an invaluable tool to examine JOD and monitor JOD prone culture sites. Using these tools and understanding the importance of the forms of *R. crassostreae* that may result in increased infectivity of the bacteria will be identified and management strategies based on bacterial sampling can be used to help minimize JOD outbreaks. This is important since the annual impact of the disease is unpredictable, and production is limited because of constraints on when seed can be safely deployed. JOD can also occur without warning in previously unaffected areas with disastrous results for growers unaccustomed to dealing with the disease. So, it is very important to understand how JOD emerges in new areas, which in turn, requires an understanding of the ecology of the causative agent. Results of this work will be summarized in a fact sheet written with the help of Walton and Rivara and provided to

all extension agents in the northeast. Results will be presented at local and national shellfish conferences and published on a websites hosted by the Marine Biological Laboratory and University of Washington. Examples of data that will be readily available include Geographic Information Systems (GIS) – based maps that show the spatial relationship of JOD prevalence and environmental conditions such as temperature and salinity. Additionally, the methods developed will be made available to other diagnostic laboratory for use in JOD evaluations.

COORDINATION WITH OTHER RESEARCH PROJECTS

There are no pending research proposals essential to the success of this proposal, however there are active and pending research projects that the current proposal will significantly complement. One of the primary research products of the current proposal will the development of a quantitative assay for detecting the disease causing agent, *R. crassostrea* in environmental samples. The PIs Smolowitz and Roberts have active research that involves environmental detection of other shellfish pathogens including MSX, Dermo [Northeastern Regional Aquaculture Center funded research], and QPX [NIH-NSF funded research]. All of these assays involve fluorescent dye based technology; therefore there is the potential to easily develop a multi-target (or multi-plex) molecular assay that would economically be able to screen environmental samples in a high-throughput manner.

Another proposal submitted to the Oyster Disease Research Program is entitled, "*Breeding and mapping disease resistance to Juvenile Oyster Disease*" submitted by Gomez-Chiarri (University of Rhode Island), Guo (Rutgers University), and Leavitt (Roger Williams University). Our combined research projects complement each other with the Gomez-Chiarri proposal concentrating on breeding disease resistant oyster and quantitative trail loci development, where our proposal focuses on the causative agent's ecology and virulence characteristics. One direct interaction of the two projects would include implementation of the quantitative assay to evaluate levels of *R. crassostrea* in disease challenge experiments. Funding of both research proposals would provide a comprehensive understanding of this emerging disease and advance the oyster industry.

REFERENCES

Barber BJ, Carnegie RB, Davis CV, Mook W (1996) Effect of timing of seed deployment on growth and mortality of oysters, *Crassostrea virginica*, affected by juvenile oyster disease (JOD). J World Aqua Soc 27(4):443-448

Barber BJ, Davis CV, Crosby MA (1998) Cultured oysters, *Crassostrea virginica*, genetically selected for fast growth in the Damariscotta River, Maine, are resistant to mortality caused by juvenile oyster disease (JOD). J Shellfish Res 17(4):1171-1175

Boardman, C.L., 2005. Host-pathogen interactions between Eastern oysters (*Crassostrea virginica*) and the bacterial agent of juvenile oyster disease (*Roseovarius crassostreae*). Master's thesis, Dept. Biochem. Microbiol. Mol. Biol., Univeristy of Maine, Orono, Maine.

Boettcher KJ, Barber BJ, Singer JT. (1999) Use of antibacterial agents To elucidate the etiology of juvenile oyster disease (JOD) in *Crassostrea virginica* and numerical dominance of an alpha-proteobacterium in JOD-affected animals. Appl Environ Microbiol. Jun;65(6):2534-9.

Boettcher KJ, Barber BJ, Singer JT. (2000) Additional evidence that juvenile oyster disease is caused by a member of the *Roseobacte*r group and colonization of nonaffected animals by *Stappia stellulata*-like strains. Appl Environ Microbiol. Sep;66(9):3924-30.

Boettcher KJ, Geaghan KK, Maloy AP, Barber BJ. (2005) Roseovarius crassostreae sp. nov., a member of the *Roseobacter* clade and the apparent cause of juvenile oyster disease (JOD) in cultured Eastern oysters. Int J Syst Evol Microbiol. Jul;55(Pt 4):1531-7.

Boettcher KJ, Maloy AP (2004) Juvenile oyster disease in the Northeast: shifting patterns of *Roseobacter*-related mortality and implications for regional management. Northeast Aquacult Conf Exposition, Manchester, NH. Conferencce proceedings available at: www.northeastaquaculture.org/agenda.htm#arc04

Boettcher KJ, Smolowitz R, Lewis EJ, Allam B, Dickerson H, Ford S, Huq A, Reece K, Rivara G, Maloy AP, Woodley CM (2006) Juvenile Oyster Disease (JOD) in *Crassostrea virginica*: synthesis of knowledge and recommendations. J Shellfish Res 25(2):683-686

Boyd, E. F. and D. L. Hartl. 1998. Chromosomal regions specific to pathogenic isolates of Escherichia coli have a phylogenetically clustered distribution. J. of Bacteriology 180: 1159-1165.

Dang, H. and C. R. Lovell. Bacterial primary colonization and early succession on surfaces in marine waters as determined by amplified rRNA gene restriction analysis and sequence analysis of 16S rRNA genes. Applied Environmental Microbiology 66: 467-475.
Dis Aquat Organ. Nov 9;67(1-2):155-62.

FAO (2006) FAO Inland Water Resources and Aquaculture Service (FIRI). c2004- .Kennedy, V. S. Cultured Aquatic Species Information Programme - *Crassostrea virginica*. Cultured Aquatic Species Fact Sheets., FAO - Rome. Updated Tue Dec 05 11:04:39 CET 2006. Available via FIGIS from: http://www.fao.org/figis/servlet/static?dom=culturespecies&xml=Crassostrea_virginica.xml

Ford SE, Borrero FJ (2001) Epizootiology and pathology of juvenile oyster disease in the eastern oyster, *Crassostrea virginica*. J Invertebr Pathol 78(3):141-154

Gast, RJ, Cushman, E, Moran, DM, Uhlinger, KR, Leavitt, D, Smolowitz R (2006) DGGE-based detection method for Quahog Parasite Unknown (QPX). Diseases of Aquatic Organisms 70:115-206

Howard, D.W., E. J. Lewis, B.J. Keller and C.S. Smith. 2004. Histological techniques for marine bivalve mollusks and crustaceans. NOAA Technicial Memorandum NOS NCCOS 5, 218 pp.

Lewis EJ (2001) Juvenile oyster disease (JOD) and management strategies: a review. Bull Natl Res Inst Aquacult, Suppl 5:101-109

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. *In press*

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. Environmental Research. *Submitted*

Maloy AP, Barber BJ, Boettcher KJ. (2005) A PCR-based diagnostic assay for the detection of *Roseovarius crassostreae* in Crassostrea virginica affected by juvenile oyster disease (JOD).

Roberts SB (2005) New molecular techniques to detect nodavirus in Atlantic cod hatcheries. Global Aquaculture Advocate 8(2): 56-57

Roberts SB and Goetz FW (2004) Quantitative real-time PCR methods for disease detection in marine fish. Aquaculture 2004, Honolulu, HI. March 4, 2004 (http://www.mbl.edu/aquaculture/nrac/Hawaii04.pdf)

Susa, M., B. Kreft, G. Wasenauer, A. Ritter, J. Hacker and R. Marre. 1996. Influence of cloned tRNA genes from a uropathogenic Escherichia coli strain on adherence to primary human renal tubular epithelial cells and nephropathogenicity in rats. Infection and Immunity 64: 5390-5394.

Current & Pending Support
Principal Investigator: Steven Roberts

Support: Active

Title: Development of genetic markers to assess disease resistance in the eastern oyster
Source of Support: USDA *(if subaward)* via: NRAC/University of Maryland
Total Award Amount: $54,066 Total Award Period: 9/1/2006-1/30/2007
Time Committed: 0.25 months

Support: Active

Title: Production of myostatin gene knockouts in zebrafish and the effects of specific myostatin interacting proteins
Source of Support: USDA *(if subaward)* via: University of Wisconsin
Total Award Amount: $195,862 Total Award Period: 1/1/2005 - 11/30/2007
Time Committed: 1.0 months

Support: Active

Title: Biological Approaches to Sensing and Analyzing Visual Scenes
Source of Support: DOD *(if subaward)* via: Battelle
Total Award Amount: $370,969 Total Award Period: 09/05/2006 – 09/04/2007
Time Committed: 1.0 months

Support: Pending

Title: The spread of lobster shell disease – genetic and social barriers
Source of Support: NOAA/RI Sea Grant *(if subaward)* via: University of Rhode Island/Sea Grant
Total Award Amount: $150,991 Total Award Period: 03/01/2007 – 12/31/2008
Time Committed: 1.0 months

Support: Pending

Title: Oyster Herpes Virus: Development of Diagnostic Probes and Understanding Pathogenesis and Transmission Dynamics
Source of Support: NOAA/Sea Grant *(if subaward)* via:
Total Award Amount: $325,474 Total Award Period: 06/01/2007 – 5/31/2009
Time Committed: 2.5 months

Support: Pending (this proposal)

Title: Characterizing the Ecology of *Roseovarius crassostreae*, the Causative Agent of Juvenile Oyster Disease
Source of Support: NOAA/Sea Grant *(if subaward)* via:
Total Award Amount: $193,926 Total Award Period: 06/01/2007 – 05/31/2009
Time Committed: 2.0 months

Current & Pending Support
Principal Investigator: Roxanna Smolowitz

Support: Current

Title: Development and application of molecular methods for detection of QPX organisms in environmental reservoirs
Source of Support: NOAA/Sea Grant *(if subaward)* via: WHOI
Total Award Amount: $18,121 Total Award Period: 03/01/2004 - 02/28/2007
Time Committed: 0.75 month

Support: Current

Title: EID Collaborative Research - Linking Marine Pathogens to Molluscan Shellfish; The Ecological Role of Marine Aggregates
Source of Support: NSF, EID - NSF 03-507 *(if subaward)* via:
Total Award Amount: $200,363 Total Award Period: 09/01/2004 - 08/31/2007
Time Committed: 1.0 month

Support: Current

Title: Effect of temperature on the infection of hard clams (Mercenaria mercenaria) by the protistian organism, Quahog Parasite Unknown
Source of Support: USDA/NRAC *(if subaward)* via:
Total Award Amount: $154,805 Total Award Period: 03/15/2005 - 03/14/2007
Time Committed: 1.5 month

Support: Current

Title: Development of genetic markers to assess disease resistance in the eastern oyster
Source of Support: USDA/NRAC *(if subaward)* via:
Total Award Amount: $54,066 Total Award Period: 09/01/2005 - 01/30/2007
Time Committed: 3.0 month

Support: Pending

Title: Cross-breeding and Field Trials of Disease-resistant Eastern Oysters, *Crassostrea virginica*
Source of Support: USDA/NRAC *(if subaward)* via: University of Maine
Total Award Amount: $158,542 Total Award Period: 11/01/2006 - 10/31/2007
Time Committed: 1.0 month

Support: Pending

Title: Environmental stress and susceptibility to shell disease – an assessment of structural deficiencies in shell formation at increased temperatures
Source of Support: RI Sea Grant College Program *(if subaward)* via: New England Aquarium
Total Award Amount: $27,588 Total Award Period: 01/02/2007 – 12/31/2008
Time Committed: 1.16 month

Support: Pending

Title: Microbiology of shell disease – environmental sources and diversity
Source of Support: RI Sea Grant College Program *(if subaward)* via: Univeristy of Louisiana
Total Award Amount: $57,793 Total Award Period: 01/02/2007 – 12/31/2008
Time Committed: 2.0 month

Support: Pending

Title: A comparison of *Perkinsus* related proteins between *Crassostrea virginica* and *Crassostrea gigas*
Source of Support: NOAA/Sea Grant *(if subaward)* via: Univeristy of Wisconsin-Milwaukee
Total Award Amount: $3,406 Total Award Period: 01/02/2007 – 12/31/2008
Time Committed: .08 month

Support: Pending (this proposal)

Title: Characterizing the Ecology of *Roseovarius crassostreae*, the Causative Agent of Juvenile Oyster Disease
Source of Support: NOAA/Sea Grant *(if subaward)* via:
Total Award Amount: $193,926 Total Award Period: 06/01/2007 – 05/31/2009
Time Committed: 3.28 months

CURRICULUM VITAE – STEVEN BEYER ROBERTS

Contact Information University of Washington
School of Aquatic and Fishery Sciences
Fisheries Teaching and Research Building
1140 NE Boat Street
Seattle, WA 98195
phone: 206.600.4495
email: sroberts@mbl.edu
sr320@u.washington.edu
web: mbl.edu/aquaculture/Roberts
fish.washington.edu/roberts

Academic Experience Ph.D. – University of Notre Dame (Notre Dame, 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

Recent Publications

Lyons MM, Lau Y-T, Carden WE, Ward JE, Roberts SB, Smolowitz R, Vallino J, Allam B. Characteristics of marine aggregates in shallow-water ecosystems: Implications for disease ecology. Environmental Research. *Submitted*

Hodgins-Davis A, Roberts SB, Cowan D, Atema J, Avolio C, Defaveri J, Gerlach G. (2006) Characterization of SSRs from the American lobster, *Homarus americanus.* Molecular Ecology Notes. *In press*

Rodgers BD, Roalson EH, Weber GM, Roberts SB, Goetz FW. (2006) A Proposed Nomenclature Consensus for the Myostatin Gene Family. AJP- Endocrinology and Metabolism. *In press*

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

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

Publications continued

Jentoft S, Topp N, Seeliger M, Malison JA, Barry TP, Held JA, Roberts SB, Goetz FW. (2005) Lack of growth enhancement by exogenous growth hormone treatment in yellow perch in four separate experiments. Aquaculture. 250:471-479

Roberts SB (2004) Lab Studies: Genes Involved with Growth, Development of Bay Scallops. Global Aquaculture Advocate. 7(3): 55-56

Ettensohn K, Biga PR, Romano C, Devlin RH, and Roberts SB. (2004) Genes Differentially Expressed in Growth Hormone Transgenic Salmon. Biological Bulletin 2004 207: 168

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

Roberts SB, Barry T, Malison J, Goetz FW. (2004) Production of a recombinantly-derived growth hormone antibody and the characterization of growth hormone levels in yellow perch. Aquaculture. Vol. 232/1-4: 591-602

Roberts SB, Goetz FW. (2003) Expressed sequence tag analysis of genes expressed in the bay scallop, *Argopecten irradians*. Biological Bulletin. 205: 227-228.

Select Presentations

•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 (*Invited*)

•Characterization of differentially expressed genes from QPX: insight into possible virulence mechanisms. National Shellfisheries Association Annual Meeting, Monterey, CA. March 28, 2006

•Overview and application of bay scallop genomics resources. National Shellfisheries Association Annual Meeting, Monterey, CA. March 27, 2006 (*Invited*)

•Developmental, stage specific gene expression in the bay scallop: interrelationship of internal and external factors. National Shellfish Association Annual Meeting, Philadelphia, PA. April 13, 2005

Scientific and Professional Organizations

American Fisheries Society
National Shellfisheries Association
Pan American Marine Biotechnology Association
Sigma Xi Scientific Research Society
World Aquaculture Society

cv ROBERTS

CURRICULUM VITAE - ROXANNA M. SMOLOWITZ

Office Address: Marine Biological Laboratory
7 MBL St.
Woods Hole, MA 02543
Ph: 508-289-7400
Email: rsmol@mbl.edu

Education:

1975-76	B.A.	Indiana State University
1977-81	D.V.M.	Purdue University
1982-84		Resident in Pathology, Angell Mem. An. Hosp., Boston, MA
1985-87		Bay Foundation Fellowship, MBL, Woods Hole, MA

Employment:

1981-82 Staff Veterinarian, Plymouth Animal Hospital, Plymouth, MA
1984-85 Pathologist, School of Veterinary Medicine, Tufts University, Boston, MA
1985-86 Clinical Instructor in Comparative Medicine, School of Veterinary Medicine, Tufts University, Boston, MA
1986-89 Laboratory Animal Veterinarian, Marine Biological Laboratory, Woods Hole, MA
1988-95 Visiting Investigator, Woods Hole Oceanographic Institution, Woods Hole, MA
1989-95 Research Associate, School of Veterinary Medicine, University of Pennsylvania at the MBL
1995-99 Senior Research Investigator in Pathology, School of Veterinary Medicine, University of Pennsylvania at the MBL
1999- Veterinarian/Aquatic Veterinary Pathologist, Marine Biological Laboratory

Veterinary Licensure: Massachusetts # 2496

Bibliography (Selected Papers):

Smolowitz, R.M., D.L. Miosky and C.L. Reinisch. 1989. Ontogeny of leukemic cells of the soft shell clam. J. Invert. Path. 52:41-51.

Wikfors, Gary H. and **Smolowitz**, R.M. Detrimental effects of a *Prorocentrum* isolate upon hard clams and bay scallops in laboratory feeding studies. Toxic Phytoplankton Blooms in the Sea. Proceedings of the Fifth International Conference on Toxic Marine Phytoplankton, Newport, RI, 28 October - 1 November 1991, T. J. Smayda and Y. Shimizu, eds. (Elsevier Science Publishers, Amsterdam, 1993) 952 pp.

Smolowitz, Roxanna M. and Carol L. Reinisch. 1993. A novel adhesion protein expressed by ciliated epithelium, hemocytes, and leukemia cells in soft-shell clams. Dev. Comp. Immunol. 17:475-481.

Smolowitz, Roxanna and Sandra E. Shumway. 1997. Possible cytotoxic effects of the dinoflagellate, *Gyrodinium aureolum*, on juvenile bivalve molluscs. Aquaculture Inter. 5: 291-300.

Ford, S., R. **Smolowitz**, L. Ragone Calve, R. Barber, and J. Kraueter. 1997. Evidence that QPX (Quahog Parasite Unknown) is not present in hatchery-produced hard clam seed. J. Shellfish. Res. 16: 519-521.

Kleinschuster, S. J., R. **Smolowitz** and J. Parent. 1998. *In Vitro* life cycle and propagation of quahog parasite unknown. J. Shellfish. Res. 17: 75-78.

Smolowitz, R., D. Leavitt and F Perkins. 1998. Observations of a Protistan disease similar to QPX in *Mercenaria mercenaria* (hard clams) fro the coast of Massachusetts. J. Invert. Pathol. 71: 9-25.

P.A. Mass,S. J. Kleinschuster, M. Dykstra, R. **Smolowitz** and J. Parent. 1999. Molecular characterization of QPX (Quahog parasite Unknown), a pathogen of *Mercenaria mercenaria*. J. Shellfish Res. 18: 561-567.

S. T. Tettelback, C. Smith, R. **Smolowitz**, K. Tetrault and S. Dumais. 1999. Evidence for fall spawning of northern bay scallops *Argopecten irradians irradians* (Lamarck 1819) in New York. J. Shellfish Res. 18: 47-58.

Sunila, I., N. A. Stokes, R. **Smolowitz**, R. C. Karney and E. M. Burreson. 2002. *Haplosporidium costale* (Seaside organism), a parasite of the eastern oyster, is present on Long Island Sound. J. Shellfish Res. 21: 113-118..

Bricelj, V.M., S.P. MacQuarrie and R. **Smolowitz**. 2004. Concentration-dependent effects of toxic and non-toxic isolates of the brown tide alga *Aureococcus anophagefferens* on growth of juvenile bivalves. Marine Ecology Progress Series 282: 101-114.

Smolowtiz, R. Gastropods. In (G. Lewbart Ed.) Invertebrate Medicine. Blackwell Publishing, Ames, Iowa. Pp.65-78.

Berkins, I. and R. **Smolowitz**. 2006. Handling of Pathological Samples from Invertebrates. In (G. Lewbart, Ed.) Invertebrate Medicine. Blackwell Publishing, Ames, Iowa. Pp.263-274.

M. Maille Lyons , Roxanna **Smolowitz**, Kevin R. Uhlinger, Rebecca J. Gast, J. Evan Ward. 2005. Lethal marine snow: Pathogen of bivalve mollusc concealed in marine aggregates. Limnol. Oceanogr. 50: 1983-1988.

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). Diseasesof Aquatic Organisms, 72:45-52.

Gast, R.J., E. Cushman, D.M. Moran, K.R. Uhlinger, D. Leavitt and R. **Smolowitz**. 2006. DGGE-based detection method for Quahog Parasite Unknown (QPX) from environmental samples and clam tissues. Diseases of Aquatic Organisms 70: 115-122.

cv SMOLOWITZ

CURRICULUM VITAE - KATHERINE J BOETTCHER

Email: kjboettcher@gmail.com
Phone: (757) 357-9069

11208 Beechwood Pointe
Smithfiled, VA 23430

EXPERIENCE AND EDUCATION:

Faculty Associate, Jan 2005 - Dec 2005	University of Maine, Orono, ME
Asst. Professor of Microbiology, Jan. 2001 - Dec 2004	University of Maine, Orono, ME
Research Assistant Professor, Oct. 2000 - Dec 2000	University of Maine, Orono, ME
Research scientist, Feb. 1999 - Sep. 2000	University of Maine, Orono, ME
Postdoctoral Research Associate, Sep. 1994 - Jan. 1999	University of Maine, Orono, ME
Ph.D. Biology, Aug. 1994	University of Southern California Los Angeles, CA
B.S. Biology, 1987	Marquette University, Milwaukee, WI

TEACHING:

University of Maine, Instructor: Bacterial Physiology, Bacterial Physiology Laboratory, General Microbiology.

University of Southern California, Teaching Assistant: General Biology, Human Biology, Cells and Organisms, Biochemistry, General Microbiology, and Human Microbiology.

AWARDS AND SCHOLARSHIPS:

Academic Scholarship: 1983-1987 (Marquette University), University Honors Certificate: 1987 (Marquette University), Teaching Assistantship: 1987-1994 (USC), Research Assistantship: 1990-1994 (USC), ARCS (Achievement Rewards for College Scientists) Foundation scholar: 1991 & 1992, Outstanding Teaching Assistant Award: Spring 1994 (USC)

PROFESSIONAL AFFILIATIONS:

American Society for Microbiology (Since 1989)
National Shellfisheries Association (Since 1996)

PROFESSIONAL SERVICE

Editorial Board – Applied and Environmental Microbiology (1/31/2003 – 12/31/2005)

Ad hoc reviewer for Applied Microbiology and Biotechnology, FEMS Microbiology Ecology, International Journal of Systematic and Evolutionary Microbiology, Plasmid.

Ad hoc reviewer of proposals for NSF, USDA, and NOAA/ SeaGrant.

PUBLICATIONS:

Maloy, A. P., B. J. Barber, and K. J. Boettcher. 2005. A PCR-based diagnostic assay for the detection of *Roseovarius crassostreae* in *Crassostrea virginica* affected by juvenile oyster disease (JOD). Dis. Aquat. Org. 67:155-162.

Boettcher, K. J., K. K. Geaghan, A. P. Maloy, and B. J. Barber. 2005. *Roseovarius crassostreae* sp. nov., a member of the *Roseobacter* clade and the apparent cause of juvenile oyster disease (JOD) in cultured Eastern oysters. Intl. J. System. Evol. Microbiol. 55:1531-1537.

Boettcher, K. J., B. J. Barber, and J. T. Singer. 2000. Additional evidence that juvenile oyster disease is caused by a member of the *Roseobacter* group, and colonization of non-affected animals by *Stappia stellulata*-like strains. Appl. Environ. Microbiol. 66:3924-3930.

Boettcher, K. J., B. J. Barber, and J. T. Singer. 1999 Use of antibacterial agents to elucidate the etiology of juvenile oyster disease (JOD) in *Crassostrea virginica*, and the numerical dominance of an α-proteobacterium in JOD-affected animals. Appl. Environ. Microbiol. 65:2534-2539.

Singer, J. T., C. Ma, and K. J. Boettcher. 1996. Overcoming a defect in generalized recombination in the marine fish pathogen *Vibrio anguillarum* 775: construction of a *recA* mutant by marker exchange. Appl. Environ. Microbiol. 62:3727-3731

Boettcher, K. J., E. G. Ruby, and M. McFall-Ngai. 1996. Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. J. Comp. Physiol. 179:65-73

Boettcher, K. J., and E. G. Ruby. 1995. Detection and quantification of *Vibrio fischeri* autoinducer from symbiotic squid light organs. J. Bacteriol. 177:1053-1058.

Boettcher, K. J., and E. G. Ruby. 1994. Occurrence of plasmid DNA in the sepiolid squid symbiont, *Vibrio fischeri*. Curr. Microbiol. 29:279-286.

Boettcher, K. J., and E. G. Ruby. 1990. Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid, *Euprymna scolopes*. J. Bacteriol. 172:3701-3706.

IN REVIEW

Maloy, A.P. S. E. Ford, R. Karney, and K. J. Boettcher. "*Roseovarius crassostreae*, the etiological agent of Juvenile Oyster Disease in *Crassostrea virginica "*Submitted to Aquaculture, Aug. 2006.

Maloy, A. P., B. J. Barber, and K. J. Boettcher. "Use of the 16S-23S rDNA internal transcribed spacer of Roseovarius crassostreae for epizootiological studies of juvenile oyster disease (JOD)" Submitted to Dis. Aquat. Org., Nov. 2006