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College of William and Mary - Virginia Institute of Marine Science

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DEVELOPMENT OF A QUANTITATIVE COMPETITIVE POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION AND QUANTITATION OF *PERKINSUS MARINUS* IN OYSTER TISSUES AND ENVIRONMENTAL WATER SAMPLES

A Thesis

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Science

---

by

Heather A. Yarnall

1998

APPROVAL SHEET

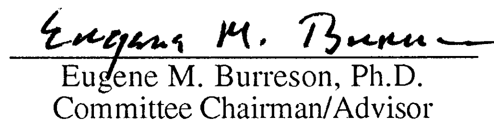
This thesis is submitted in partial fulfillment of

the requirements for the degree of

Master of Science

Heather Ann Yarnall

Approved, January 1998

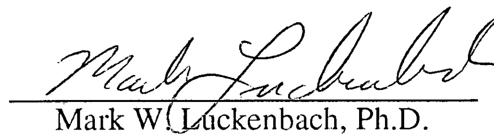


Eugene M. Burreson, Ph.D.
Committee Chairman/Advisor

Kimberly S. Reece, Ph.D.

Stephen L. Kaattari, Ph.D.

Roger L. Mann, Ph.D.



Mark W. Luckenbach, Ph.D.

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ABSTRACT

Ray's fluid thioglycollate medium (RFTM) culture methods are used for the routine monitoring of *Perkinsus marinus* infections in oyster tissues. Some drawbacks of RFTM are that the assays detect all *Perkinsus* sp. and sensitive quantitation requires the use of the entire oyster tissue. Molecular methods, especially the polymerase chain reaction (PCR), are species specific and exceptionally sensitive for diagnosis. PCR has often been employed to detect a pathogen but has typically been limited to a presence or absence result. Quantitative competitive PCR (QCPCR), however, relies on an internal standard in each reaction to precisely quantitate the amount of target DNA originally present. This work focused on developing a QCPCR assay for *P. marinus* quantitation in oyster tissues as well as in environmental water samples.

Perkinsus marinus specific primers (PER-18S and PER-ITS) were developed from the ribosomal small subunit gene and the first internal transcribed spacer region. The primers amplified DNA isolated from *P. marinus* but not from *P. atlanticus,* the eastern oyster *Crassostrea virginica*, or the dinoflagellates *Peridinium sp.*, *Gymnodinium sp.*, and *Amphidinium sp.* A mutagenic primer (ITS-MUT) was used to create a competitor plasmid identical to the *P. marinus* target DNA except for a 13 bp deletion. The target *P. marinus* DNA and the competitor molecule amplified with equivalent efficiencies with the PER-18S and PER-ITS primers. The use of the LiCor DNA 400L automated sequencer allowed separation of the two amplified DNA species in each reaction. The sequencer software provided precise determination of the equivalence point in each series of PCR reactions.

Oysters were sampled from the James River in Virginia (n=25), and each oyster was processed by five *P. marinus* diagnostic methods: Ray's FTM assay, FTM hemolymph assay, body burden assay, QCPCR with gill and mantle tissue, and QCPCR with hemolymph. As little as 0.005 fg of *P. marinus* DNA in approximately 0.4 µg of background oyster tissue DNA could be quantitated. A standard curve, prepared by performing QCPCR on DNA isolated from enumerated *P. marinus* cultured cells in background oyster tissue, allowed the calculation of the number of *P. marinus* cells/g of oyster tissue in the naturally infected oyster samples. By this method, as few as 2 *P. marinus* cells could be quantitated. Overall, regression analysis showed that the QCPCR methods were effective in diagnosing *P. marinus* infection in oyster tissues. In fact, the QCPCR hemolymph assay diagnosed 24 of 25 oysters as being infected as did the "gold standard" body burden assay.

The QCPCR assay was also used to quantitate the *P. marinus* DNA present in water samples obtained from the lower York River in September 1997 and October 1997. A modified body burden protocol was used for triplicate water samples although no *P. marinus* cells were found in the FTM after incubation. The QCPCR assay, however, detected *P. marinus* DNA in water samples from both sampling dates. Thus, QCPCR can be applied for the sensitive detection and quantitation of *P. marinus* cells in water samples in order to conduct essential experiments on waterborne stages of this parasite.

Development of a Quantitative Competitive Polymerase Chain Reaction Assay for the Detection and Quantitation of *Perkinsus marinus* in Oyster Tissues and Environmental Water Samples

INTRODUCTION

Perkinsus marinus

Perkinsus marinus has been a significant cause of mortality of the eastern oyster *Crassostrea virginica* along the east coast of the United States from the 1950s to the present (Andrews, 1988; Burreson and Ragone Calvo, 1996). This protozoan parasite was first discovered in the Gulf of Mexico by Mackin et al. (1950) and was originally classified as *Dermocystidium marinum* due to the signet ring appearance of the mature trophozoite. Mackin and Ray (1966) changed the name to *Labyrinthomyxa marina* based on the observed amoeboid stages in the oyster which moved by gliding. Perkins (1976) later noted an apical complex in the flagellated zoospores of the protozoan. This discovery prompted the reclassification of the parasite as *Perkinsus marinus* in the phylum Apicomplexa (Levine, 1978). Vivier (1982), however, argued against this classification by emphasizing that the trilaminar pellicle and the micropores are not unique to the apicomplexans and that the conoid resembles that of the ciliates and flagellates as opposed to the sporozoans. This morphological evidence has spurred debates on the placement of *Perkinsus marinus* within the apicomplexans. Recently, some researchers have suggested that *P. marinus* may be more closely related to the dinoflagellates than to the apicomplexans based on molecular phylogenetic analyses (Goggin and Barker, 1993; Fong et al., 1993; Goggin, 1994; Siddall et al., 1995; Flores et al., 1996, Reece et al., 1997b, Siddall et al., 1997).

Historically, *P. marinus* has infected oysters in the Gulf of Mexico from Florida to Mexico as well as along the eastern seaboard of the United States from Florida northward

to the Chesapeake Bay (Mackin, 1962; Andrews and Hewatt, 1957; Andrews, 1988; Burreson et al., 1994). In the late 1980s and early 1990s, *P. marinus* spread up the estuary to regions of typically lower salinity waters in the Chesapeake Bay and the Delaware Bay and extended its distribution as far north as Maine (Ford, 1996). The northern range extension of this parasite may have been a result of repeated introductions of infected oysters, a change in the genetic structure of the host or the parasite, a change to more favorable environmental conditions, or some combination of these factors (Ford, 1996).

Environmental factors such as salinity and temperature have played critical roles in determining the range of *P. marinus* as well as the severity of the epizootics. Increasing salinity is positively correlated with prevalence and intensity of the parasite (Andrews, 1988; Mackin, 1956; Craig et al., 1989). Furthermore, early research showed that low salinity actually retarded the development of *P. marinus* (Ray, 1954). Ragone and Burreson (1993) reported that the critical minimum salinity range for *P. marinus* infection is 9 to 12 ppt and that the pathogen is less virulent at salinities less than 9 ppt. Four consecutive drought years in the late 1980s, along with warm winters, allowed the spread of *P. marinus* up the estuary to regions of previously low salinities (Burreson and Ragone Calvo, 1996). Temperature affects the geographical distribution of *P. marinus* (Ray and Mackin, 1954; Andrews and Hewatt, 1957; Burreson and Ragone Calvo, 1996) with the minimum winter temperature determining the northern limit of this pathogen (Andrews, 1988). The spread of *P. marinus* up bay was possible because warmer winter temperatures in the early 1990s lessened the overwintering parasite loss as well as accelerated the development of lethal infections in early spring (Burreson and Ragone Calvo, 1996; Ford, 1996). Temperature, however, plays a more significant role in the seasonal periodicity of *P. marinus* (Burreson and Ragone Calvo, 1996). New

infections are acquired in July to October with a peak in August shortly after annual maximum temperatures and during the period of maximum oyster mortality.

Transmission of *P. marinus* has been reported to occur from infected oysters to uninfected oysters via the water column (Ray and Mackin, 1954). All identified life stages including the meront, prezoosporangia, and biflagellated zoospore have been shown to cause infection in the eastern oyster (Chu, 1996) although Volety and Chu (1994) determined that meronts are more infective than prezoosporangia. In studies to determine the necessary dose of infective cells to initiate an infection, it was found that $10\text{-}10^2$ *P. marinus* cells were required for infection (Chu, 1996). Oysters are filter-feeding organisms and *P. marinus* has been found in the water column at abundances of 3000 to 19000 cells l^{-1} of water in the warm summer months (Dungan and Roberson, 1993). Thus, *P. marinus* transmission can clearly be successful in the water column from oyster to oyster.

P. marinus Detection and Diagnosis

Presently, investigators rely on methods of Ray's fluid thioglycollate medium (RFTM) culture of *Perkinsus marinus* trophozoites from oyster tissue (rectum, gill, and mantle) for the diagnosis of infected oysters (Ray, 1952). Choi et al. (1989) developed a protocol to digest oyster tissue in RFTM cultures by using sodium hydroxide which enables the isolation of the enlarged *P. marinus* cells. As a result, body burden assays in whole oyster homogenates (Bushek et al., 1994) and hemolymph assays (Gauthier and Fisher, 1990) were developed based on Ray's technique.

In order to use Ray's fluid thioglycollate medium culture as a reliable method of detection of *P. marinus,* several assumptions must be made (Bushek et al., 1994; Fisher and Oliver, 1996). All life stages of the parasite must be retrieved from the host and they must be detectable by the diagnostic procedures used. This assumption involves the

enlargement of all *P. marinus* cells and specific staining of them by Lugol's iodine. Furthermore, quantitation requires accurate counting and the use of a subjective semi-quantitative scale which was developed by Mackin (1962) and modified by Craig et al. (1989). It is also assumed that the number of parasites remains constant after collection of the sample and through the incubation period in RFTM. Another important assumption for the standard tissue assay and the hemolymph assay is that the assay represents the total tissue distribution of the infection. Quantitative RFTM diagnosis using whole oysters may overcome the limitations of several of these assumptions by enumerating the total number of parasites in the oyster (Bushek et al., 1994; Fisher and Oliver, 1996). The RFTM culture diagnostic methods are relatively simple to perform; however, intensities of infection are subjective (Mackin 1962; Craig et al., 1989), and the method is often not sensitive enough to detect extremely light infections.

Attempts have been made to produce antibodies which specifically recognize *P. marinus* . A particulate hypnospore immunogen was isolated from infected oyster hemolymph after incubation in RFTM and utilized for the production of antisera (Dungan and Roberson, 1993). The resulting antibodies were found to label the cytoplasm as well as the nucleus of the mature trophozoite cell. Oyster tissue was not labeled nor were *Haplosporidium nelsoni* or *Dermocystidium spp.* The antibodies also had affinities for *P. olseni* and *P. atlanticus* but not for *P. karlssoni* (Dungan and Roberson, 1993). These polyclonal antibodies have been used for detection of *P. marinus* although the ultimate specificity of the antibodies has yet to be determined. By using this alternative method, Ragone Calvo and Burreson (1994) were able to detect light infections in oysters which were not detected using standard and hemolymph RFTM culture methods. Furthermore, a flow cytometric immunoassay utilizing the *P. marinus* antibodies has been utilized to monitor the annual abundances of the pathogen in the water column (Ragone Calvo et al., 1995).

Molecular techniques have recently been the focus of a new approach for the diagnosis of the presence of this pathogen in all of its life stages in both oyster tissues and alternative hosts as well as in the environment. Sequence data from the small subunit (SSU) ribosomal RNA gene (Fong et al., 1993; Goggin and Barker, 1993) as well as from the two internal transcribed spacers (ITS) and the 5S rRNA gene (Goggin, 1994) of *P. marinus* have been reported. These nucleotide sequences have regions which are specific to *P. marinus* and thus can be used to develop specific detection assays for the oyster parasite. A noncoding domain of *P. marinus* DNA was used to develop a semi-quantitative polymerase chain reaction (PCR) assay for estimating the level of infection in oysters (Marsh et al., 1995).

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a useful technique which was invented by Kary Mullis (Mullis et al., 1986; Mullis and Faloona, 1987). This molecular tool allows the specific amplification of a small region of DNA from a target organism with high sensitivity despite the presence of contaminating DNA from other organisms (Darnell et al., 1990). PCR utilizes paired primers which bind specifically to a region in the genomic DNA in an individual species based on their specific complementary nucleotide sequence. The primers allow the amplification of the desired target region of DNA which is flanked by these primers. Simply, this method involves temperature cycling of the target DNA, primers, and a thermal-stable DNA polymerase enzyme through many phases of heat denaturation of the genomic DNA, annealing of the primers to the specific region of DNA, and finally extension of the primers by the DNA polymerase. In subsequent cycles, each newly synthesized DNA fragment can serve as a template in addition to the original DNA template. Ultimately, the result is an exponential increase in the copy

number of the specific DNA fragment which can then be visualized by gel electrophoresis (Saiki, 1990).

Disease Diagnosis by Molecular Techniques

PCR techniques have been utilized for the detection of pathogens in the aquatic environment as well as in the host. DNA-based PCR assays were used to detect *Hematodinium spp.* dinoflagellates in decapod crustaceans (Hudson and Adlard, 1994). A successful DNA-based PCR assay was used to detect *Renibacterium salmoninarum* in individual salmonid eggs (Brown et al., 1994). Furthermore, this method proved to be more sensitive and more specific than both of the previously accepted methods, the enzyme-linked immunosorbent assay and the fluorescent antibody test (Brown et al., 1995). Barlough et al. (1995) employed a nested polymerase chain reaction involving two sets of primers to detect genomic DNA from the microsporidian parasite *Enterocytozoon salmonis* in chinook salmon.

An RNA-based assay which involved reverse transcription and subsequent PCR of a nucleoprotein gene has served as the basis for the detection of infectious hematopoietic necrosis virus (IHNV) in salmonids (Arakawa et al., 1990). A similar technique was used to amplify part of the coat protein gene of striped jack nervous necrosis virus (SJNNV) and for diagnosis of larval striped jack with viral nervous necrosis (Nishizawa et al., 1994).

Several PCR assays for the detection of oyster protozoan pathogens have also been developed. The protozoan *P. marinus* can be detected in oyster tissues using a diagnostic PCR assay which amplifies a region of DNA within a noncoding domain (Marsh et al., 1995). PCR primers have also been designed to amplify a region of the small subunit ribosomal DNA in *Haplosporidium nelsoni*, another protozoan oyster parasite (Stokes et al., 1995). In this assay, PCR was a superior diagnostic technique when compared to

typical histological examination of oyster tissues. In addition, PCR was used to detect *H. costale* in samples of DNA isolated from oysters infected with this parasite (Ko et al., 1995).

Quantitative PCR

PCR has been used as a semi-quantitative method since its development in the 1980s. For absolute quantitation, Southern blots for DNA and Northern blots for RNA have historically been the accepted molecular methods (Ferre, 1992). Quantitation of nucleic acids, however, is within the range of capabilities of the polymerase chain reaction and the sensitivity of this method is excellent. For example, quantitative PCR has been used to detect HIV to the limits of a single viral genome in a background of 5×10^4 cells (Piatak et al., 1993). This quantitative method is moving to the forefront of diagnostic science due to its ability to detect extremely low copy number DNAs or RNAs in crude cell lysate preps (Zimmermann et al., 1994).

The initial exponential increase in product serves as the major limitation for the use of PCR as a quantitative method. Any variation between replicate samples leads to changes in amplification efficiency which may, in turn, cause dramatic changes in the product yield (Gilliland et al., 1990b). This may obscure differences in the amounts of DNA or RNA which are being measured and thus preclude quantitation (Siebert and Larrick, 1993). Thus, interassay variability must be controlled in quantitation experiments. Under ideal amplification conditions, the target DNA is amplified at a nearly constant exponential rate throughout the exponential phase. Following this phase, the synthesis rate diminishes and the plateau phase exhibits a decreased product accumulation (Soong and Arnheim, 1995). This decrease may result from a limiting concentration or thermal inactivation of the DNA polymerase, a reduction in denaturation efficiency, a decrease in the primer to template ratio, or a progressive decrease in the efficiency of the primer-

template annealing as a result of increasing reannealing between the product strands and the template (Soong and Arnheim, 1995). Most accurate quantitation occurs within the exponential phase since the product amount will reflect the amount of starting target DNA. Several additional factors affect the quantitative power of PCR (Ferre, 1992). Initially, the development of an assay for quantitating nucleic acids using PCR must include the optimization of the assay conditions. Enzyme concentration, deoxynucleotide triphosphates, buffer system, denaturation time and temperature, annealing temperature, extension time, and cycle number--these factors must be optimized in order to accurately quantitate nucleic acids using PCR (Innis and Gelfand, 1990). In the case of analyses of soil, sediment, or water samples, the conditions of the PCR must also be optimized in order to counteract the effects of the contaminating inhibitors of *Taq* DNA polymerase which are often present in environmental samples. Such additional optimization allowed the development of a quantitative competitive polymerase chain reaction assay to detect *Pseudomonas* cells in seawater (Leser, 1995).

A brief overview of quantitative PCR methods listed five approaches to quantitating nucleic acids using PCR: semi-quantitative methods, Poisson dilution, coamplification of target DNA using independent internal standards, competitive coamplification of target DNA with internal standards, and non-competitive amplification with external standards (Soong and Arnheim, 1995). The two most employed methods of quantitative polymerase chain reaction, however, are the competitive and the noncompetitive methods. In the competitive method, the standard and the target DNA are present in the same reaction tube and thus compete for the same DNA polymerase and deoxynucleotide triphosphates during the amplification (Sur et al., 1995). Non-competitive PCR involves the amplification of the target DNA and a dilution series of the standard DNA in separate reaction tubes (Sur et al., 1995). These two methods gave comparable results for the interleukin (IL-5) gene DNA, although the competitive method yielded results that were

more similar to the actual values for the amount of target DNA in the sample (Sur et al, 1995).

Internal competitive standards for quantitative PCR typically have sequences that are homologous to the target nucleic acids. The target and the competitor, therefore, amplify with the same kinetics (Ferre, 1992). The competitive standard is similar to the target DNA and amplifies with the target sequence primers or slightly modified primers. Also, the differences between target and competitor can easily be detected after amplification. Often a constructed plasmid serves as the competitive standard for quantitative PCR; however, plasmid DNA amplifies with a greater efficiency than does genomic DNA. Prior to PCR, the genomic DNA may be digested with a restriction endonuclease in order to minimize differences in amplification efficiency between the target and standard DNA by rendering the genomic DNA more accessible to the DNA polymerase used in PCR.

Internal competitive standards are used for quantitation of the absolute amount of target nucleic acids, while external standards are often used for relative amounts of nucleic acids (Soong and Arnheim, 1995; Siebert and Larrick, 1993). Sur et al. (1995) suggested that absolute quantitation yields results that only apply under the given conditions because values may change given other conditions. Internal competitive standards eliminate error resulting from tube to tube variation because both the standard and the target will be equally affected by the internal PCR conditions (Gilliland et al, 1990a; Soong and Arnheim, 1995). Furthermore, competitive PCR overcomes the need to perform quantitation in the exponential phase (Siebert and Larrick, 1993). Both the standard and the target are equally affected by the changing amplification parameters and thus, the reactions are cycle-independent (Soong and Arnheim, 1995). Therefore, temperature cycling into the plateau phase does not interfere with quantitation and even increases the sensitivity of the assay (Morrison and Gannon, 1994). The finding that the plateau phase results not from limiting reaction components but as a feature of the reaction

itself is important for quantitation for two reasons. First, a constant maximum of products is obtained even after amplification of varying amounts of a single target, and secondly, the coamplification of different concentrations of different targets will result in retention of the initial proportions even in the plateau phase (Morrison and Gannon, 1994). In competitive PCR, the inhibitors in the reaction equally affect the amplification of both the target and the standard DNA and thus minimize the effect of inhibitors on the quantitation process. As a result total DNA from infected cells can be used directly in quantitative competitive PCR (QCPCR) (Piatak et al., 1993).

Ultimately, quantitation by competitive PCR involves a set of reactions which include a constant aliquot of unknown DNA and a series of dilutions of known concentrations of the standard competitor DNA. At the point where the molar amounts of the two products are equivalent, the amount of original target DNA present in the sample is equivalent to the amount of standard added initially (Piatak et al., 1993). Thus, quantitation of the unknown DNA is based upon the attainment of an equivalence point at a known concentration of standard competitor DNA.

DNA standards for competitive PCR have been constructed by the insertion or deletion of a restriction enzyme recognition site by site mutagenesis (Gilliland et al, 1990a). After amplification, these products can be distinguished by appropriate enzyme digestion and gel electrophoresis. Using restriction endonuclease digestions, however, is not an optimal means for distinguishing the target from the competitor since the digestion may proceed with variable and unpredictable efficiency (Clementi et al., 1993). As a result, altering the size of the competitor molecule provides a preferable means for generating the internal standard. Siebert and Larrick (1993) created similar non-homologous standards by ligation of a gene fragment to the complementary primer sequences. The capabilities of automated sequencers to detect a one nucleotide difference in sequences have been applied to QCPCR (Porcher et al., 1992; Lu et al, 1994; Cottrez et al., 1994; Thiery et

al., 1995; Repp et al, 1995). Internal standards were generated by PCR amplification using altered forms of the original target DNA primers. The primers were designed by overlapping the sequence of the original primer, linking this sequence to five nucleotides within the gene and thus creating a six base pair gap in the PCR product (Thiery et al., 1995). The difference was sufficient to be detected using an automated sequencer but was relatively insignificant to the reaction kinetics in the PCRs with the unknown target DNA and the competitor DNA (Thiery et al., 1995).

Finally, the credibility of PCR for the quantitation of nucleic acids relies on the assessment of the limits of the assay (Ferre, 1992). The sensitivity, specificity, accuracy, precision, and reproducibility of the reaction must be determined in order to interpret the results. Precision is the most important measurement of interassay variability while reproducibility is the most important measurement of intraassay variability (Ferre, 1992). Using internal competitive standards, however, eliminates many of the effects of variability in the accurate quantitation of nucleic acids.

Objectives

The primary objective of this thesis research was to develop a specific and sensitive quantitative competitive polymerase chain reaction assay for *Perkinsus marinus*. This study examined the relationship between the QCPCR assays and the more traditional fluid thioglycollate media methods. Furthermore, the assay was employed to quantitate *P. marinus* in environmental water samples.

MATERIALS AND METHODS

Perkinsus marinus-specific primers

Primers for this work were derived from the published *P. marinus* DNA sequences for the ribosomal small subunit (SSU or 18S) gene (Fong et al., 1993) and the adjacent internal transcribed spacer (ITS-1) sequence (Goggin, 1994). These primers, designated PER-18S and PER-ITS, specifically amplified a 1210 base pair fragment of DNA from within the SSU rRNA gene to within the ITS-1 of the ribosomal DNA region. Figure 1 shows the DNA sequence of the two primers as well as their location within the small subunit rRNA gene and the internal transcribed region. The primer designated PER-ITS was chosen from within the ITS-1 sequence because the ITS region (ITS-1 and ITS-2 together) was previously found to be specific (13% difference) to *P. marinus* in relation to other species of the genus *Perkinsus* (Goggin, 1994). These two primers satisfied the following general requirements for primers suitable for PCR. Efficient primers are typically 18 to 28 nucleotides in length and have a 50 to 60 % guanidine and cytosine (G+C) concentration (Innis and Gelfand, 1990). Also, the primer sequences did not have complementarity at the 3' ends of the primer pairs, runs of G's or C's at the 3' ends, or palindromic sequences within the primers (Innis and Gelfand, 1990).

Isolation of DNA from cultured cells

Perkinsus marinus cultures were obtained from Dr. Aswani Volety and from Dr. Jerome LaPeyre (Virginia Institute of Marine Science, Gloucester Point, VA). *P.*

Figure 1. Sequence of the *Perkinsus marinus* small subunit rRNA gene (*SSU*) and the first internal transcribed spacer (*ITS1*). The 1210 bp amplified region is in larger print with the primer sequences PER-18S and PER-ITS underlined and ITS-MUT in bold type.

SSU

AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AAGTATAAGC TTTAAACGGC GAAACTGCGA
ATGGCTCATT AAAACAGTTA TAGTTTATTT GGTGATCGAT TACTATTTGG ATAACCGTAG TAATTCTAGA GCTAATACAT GCGTCAAGGC
CCGACTTCGG AAGGGCTGCG TTTATTAGAT ACAGAACCAA CCTAGCTCCG CCTAGTCCTT GTTGGTGATT CATAATAACC CGGCGAATGA
CGCTTGTCGC GATGGACCAT TCAAGTTTCT GACCTATCAG CTATGGACGG TAGGGTATTG GCCTACCGTG GCGTTGACGG GTAACGGGGA
ATTAGGGTTC GATTCCGGAG AGGGAGCCTG AGAAACGGCT ACCACATCTA AGGAAGGCAG CAAGGCGCGC AAATTACCCA ATCCTGATAC
AGAGAGGTAG TGACAAGAAA TAACAATACA GGGCAATTCT GTCTTGTAAT TGGAATGAGT AGATTTTAAA TCTCTTTACG AGTATCAATT
GGAGGGCAAG TCTGGTGCCA GCAGCCGCGT AATTCCAGCT CCAATAGCGT ATATTAAAGT TGTTGCGTTA AAAGCTCGT AGTTGGATTT
CTGCCTTGGG CGACCGATCC ACCTTT

<u>CCTA CGGGATTGGT TGGTATCAG</u>G

TTTGACCTTG GCTTTTTCTT GGGATTCGTG CCCACGTACT TAACTGTGCG TTGACCGTGT TCCAAGACTT

TTACTTTGAG GAAATTAGAG TGTTTCAAGC AGGCTTATGC CATGAATACA TTAGCATGGA ATAATAGGAT

ATGACTTCGG TCATATTTTG TTGGTTTCTA GGACTGAAGT AATGATTAAT AGGGACAGTC GGGGGCATTC

GTATTTAACT GTCAGAGGTG AATTCTTGGA TTTGTTAAGA CGAACTACTG CGAAACAGTT TGCCAAGGAT

GTTTTCATTG ATCAAGAACG AAAGTTAGGG GATCGAAGAC GATCAGATAC CGTCCTAGTC TTAACCATAA

ACTATGCCGA CTAGGGATTG GGGTCGTTA ATTTTAGACG CCCTCAGCAC CTCGTGAGAA ATCAAAGTCT

TTGGGTTCCG GGGGGAGTAT GGTCGCAAGG CTGAAACTTA AAGGAATTGA CGGAAGGGCA CCACCAGGAG

TGGAGCCTGC GCTTAATTTG ATTCAACACG GGAAAACTCA CCAGGTCCAG ACATAGGAAG GATTGACAGA

TTGATAGCTC TTTCTTGATT CTATGGGTGG TGGTGCATGG CCGTTCTTAG TTGGTGGAGT GATTTGTCTG

GTTAATTCCG TTAACGAACG AGACCTTAAC CTGCTAAATA GTTGCGCGAA ACTTTATGTT TCGTCGCTAC

TTCTTAGAGG GACTTTGTGT ATTTAACACA AGGAAGCTTG AGGCAATAAC AGGTCTGTGA TGCCCTAGAT

GTCTGGGCTG CACGCGCCCT ACACTGACAC GATCAACAAG TATTTCCTTG CCCGGTAGGG TTAGGGTAAT

CTTTTGAAAT CGTGTCGTGC TAGGGATAGA CGATTGCAAT TATTCGTCTT CAACGAGGAA TTCCTAGTAA

ATGCAAGTCA TCAGCTTGCG TTGATTACGT CCCTGCCCTT TGTACACACG CCGTCGCTCC TACCGATTGA

GTGATCCGGT GAGCTGTCCG GACTGCATTA GTTCAGTTTC TGTTCTCTTC GCGGGAAGTT

ITS1

CTGCAAACCT TATCACTTAG AGGAAGGAGA AGTCGTAACA AGGTTTCCGT AGGTGAACCT GCGGAAGGAT
CATTCACACC

GATTCATTCT CTGAGAAAC**C AGCGGTCTC**G CTCTCTCTTG CT**<u>CTTTTGTT AGAGAGTTGC</u>**

<u>GAGATG</u> GGAT CCCCGCTTTG TTTGGATCCC CCCACCTTAA CTTGTTAAGG TGATTAATTC CTATGAACCA TTGTACTAGT CATAGTATCC

AAATCCAATT TTGGATTTTG GTATTTCAAA ACGAAATTCCA

marinus cells were maintained in culture according to the methods of Gauthier and Vasta (1993) or LaPeyre et al. (1993) respectively. DNA was isolated from these cells as follows. Cells were pelleted by centrifugation and washed twice with TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0). Then 0.5 ml of lysing solution (50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 1% Sarkosyl, 0.5 mg/ml Proteinase K) was added to each tube. Samples were incubated overnight at 50 °C on a rotator. The lysing efficiency of *P. marinus* cultured cells by this method was found to be 99.9%. As a result, no correction for possible unlysed cells in a sample was necessary in subsequent quantitative analysis. After incubation, the samples were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) 1-2 times as needed to remove cellular protein and then with chloroform:isoamyl alcohol (24:1) one time. DNA was precipitated with 0.04 volumes of 5M NaCl and 2 volumes of 95% EtOH and stored overnight at -20 °C. Nucleic acids were pelleted by centrifugation, washed with 70% EtOH, and allowed to air dry. The DNA was resuspended in TE buffer. Genomic DNA was digested for 2 hours at 37 °C with *Xba*I, a restriction endonuclease, prior to amplification in order to cut up the DNA into fragments which are more accessible to the polymerase during PCR. *Xba*I does not cut within the 1210 base pair PCR product. The restriction endonuclease was inactivated by incubation at 65 °C for 20 minutes. The DNA was stored at 4 °C until use in PCR reactions.

Similarly, cultured *Perkinsus atlanticus* cells were obtained from Dr. Stephen Kleinschuster (Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ). Although there has been some debate as to the identity of these cells, the cultured cells have been identified as *P. atlanticus* (Kleinschuster et al., 1994). DNA was isolated according to the method described above for cultured *P. marinus* cells.

DNA from various *P. marinus* isolates was obtained from Dr. Kimberly Reece (Virginia Institute of Marine Science, Gloucester Point, VA). The *in vitro* cultures from

Virginia (VA-1), Connecticut (CT-1), Louisiana (LA-1), and Texas (TX-1) were isolated by D. Bushek (Bushek, 1994). The South Carolina (SC-1) culture was isolated by D. Bushek (Reece et al., 1997a). The DNA was isolated as described above for other cultured *P. marinus* isolates.

Recent phylogenetic evidence suggests that *P. marinus* is most closely related to the dinoflagellates (Goggin and Barker, 1993; Fong et al., 1993; Goggin, 1994; Siddall et al., 1995; Flores et al., 1996; Reece et al., 1997b; Siddall et al., 1997). As a result, DNA was isolated from cultured *Amphidinium* sp., *Peridinium* sp., and *Gymnodinium* sp. Dinoflagellate cultures were obtained from Carolina Biological Supply Company. DNA was isolated from these cells using a microwave lysis preparation protocol developed by Goodwin and Lee (1993) followed by hexadecyltrimethyl ammonium bromide (CTAB) extractions (Ausubel et al., 1988). Briefly, cells were pelleted by centrifugation, washed in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), and resuspended in 100 µl microwave lysis preparation buffer (50 mM Tris, pH 7.2, 50 mM EDTA, 3% sodium dodecyl sulfate (SDS), 1% 2- mercaptoethanol). The microcentrifuge tubes were covered with parafilm and a small hole was punched in the parafilm. The tubes were microwaved on high power until the onset of boiling after approximately 1 minute. The samples were incubated for 10 minutes at 80 °C. After incubation, 5 M NaCl was added to a final concentration of 0.7 M. One-tenth sample volume of 10% CTAB in 0.7 M NaCl was added to each sample, and the samples were incubated for 10 minutes at 65 °C. To remove both the contaminants and the CTAB, the sample was extracted with an equal volume of chloroform:isoamyl alcohol (24:1). Again, 1/10 volume of CTAB was added to the aqueous layer and incubated for 10 minutes at 65 °C. This step was followed by a chloroform:isoamyl alcohol (24:1) extraction. The sample was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and then again with chloroform:isoamyl alcohol (24:1). The DNA in the final aqueous layer was

precipitated with the addition of 0.6 volumes of isopropanol and was stored overnight at -20 °C. Nucleic acids were pelleted by centrifugation and washed in 70% EtOH. After air drying, DNA was resuspended in TE buffer and stored at 4 °C. This procedure was followed by ethidium bromide/high salt extractions (Stemmer, 1991). Briefly, 150 µg ethidium bromide and 140 µl 7.5 M ammonium acetate were added to the 250 µl of resuspended DNA. The sample was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and then with an equal volume of chloroform:isoamyl alcohol (24:1). The DNA was precipitated with 2 volumes 95% ethanol and 10 µg glycogen overnight at -20 °C. The DNA was pelleted by centrifugation and resuspended in TE buffer.

Preparation of scallop DNA spiked with *P. marinus* DNA

DNA from *Argopecten irradians* tissue was obtained from Jan McDowell (Virginia Institute of Marine Science, Gloucester Point, VA). DNA was isolated as described above for cultured *Perkinsus marinus* cells. Following isolation, DNA was purified by CTAB extractions as described above for the dinoflagellate culture DNA isolations. Scallop DNA was spiked with DNA isolated from cultured *P. marinus* cells. The DNA mixture was digested with *Xba*I for 2 hours at 37 °C and then the enzyme was inactivated at 65 °C for 20 minutes. The final concentration of *P. marinus* DNA in the background of *A. irradians* DNA was 10 ng/µl, 500 pg/µl, or 10 pg/µl.

PCR with actin primers

DNA isolated from *Crassostrea virginica*, *Argopecten irradians*, *Amphidinium* sp., *Gymnodinium* sp., and *Peridinium* sp. was amplified with a pair of "universal" actin primers designed by G. Warr (Medical University of South Carolina) and M. Wilson (Mississippi State University). Each reaction was made from 1 µl DNA and 24 µl of

master mix. Reagent concentrations were as follows: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 12.5 pmoles of each primer, 200 µM each of dATP, dCTP, dGTP, and dTTP, bovine serum albumin (BSA, final concentration of 400 ng/µl), and 1 unit of Ampli*Taq* DNA polymerase (Perkin Elmer) in a total reaction volume of 25 µl. Reactions were subjected to temperature cycling as follows: initial denaturation step of 4 minutes at 95 °C, 40 cycles of 1 minute at 95 °C, 1 minute at 45 °C, and 3 minutes at 65 °C, and a final extension step of 5 minutes at 65 °C.

PCR with PER-18S and PER-ITS

Reaction conditions for the PCR with these two primers were optimized as it has been demonstrated that factors such as polymerase concentration, deoxynucleotide triphosphate concentration, buffer system, denaturation time and temperature, annealing temperature, extension time, and cycle number affect the specificity and sensitivity of the PCR (Ehrlich et al., 1991). PCR reactions were made from a master mix and 1 µl of DNA. The master mix contained reaction buffer (Invitrogen 5x Buffer C: 300 mM Tris-HCl pH 8.5, 75 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$), 12.5 pmoles of each primer, 200 µM each of dATP, dCTP, dGTP, and dTTP, BSA (final concentration of 400 ng/µl), and 1 unit of Ampli*Taq* DNA polymerase (Perkin Elmer) in a total reaction volume of 25 µl. BSA has been shown to overcome inhibition of the *Taq* polymerase by substances commonly found in environmental samples (Kreader, 1996). Samples were denatured initially at 94 °C for 5 minutes. Then, the samples were cycled 35 times in a DeltaCycler II thermal cycler (Ericomp) at 94 °C for 1 minute, 59 °C for 1 minute, and 72 °C for 3 minutes. A final extension period of 5 minutes was run at 72 °C. The annealing temperature of 59 °C was found to yield one product band at 1210 bp as determined by comparison with DNA size standards on an agarose gel. The LiCor automated sequencer could adequately detect the product bands produced under these conditions.

Construction of competitor DNA

A competitor DNA molecule was constructed. First, a new primer (ITS-MUT) was chosen and synthesized (Genosys). This modified primer contained the entire sequence of the PER-ITS primer; however, the ITS-MUT primer created a gap of thirteen nucleotides in the sequence to be amplified by way of a link to a region of sequence several bases upstream within the ITS-1 region. The location and sequence of the ITS-MUT primer within the SSU rRNA gene is shown in Figure 1. Figure 2 illustrates the modified primer sequence in relation to the original primer sequence. Amplification with this ITS-MUT primer and the PER-18S primer was carried out according to the protocol described above except that the annealing temperature was changed to 50 °C. The resulting 1197 bp PCR product of PER-18S and ITS-MUT was homologous to the PCR product from the original primer pair PER-18S and PER-ITS except for this thirteen base pair deletion. The size difference between the modified and original PCR products was sufficient to be detected on an acrylamide gel using an automated sequencer. Furthermore, it has been shown that competitor molecules, which are less than 5% different in size but otherwise homologous to the target sequence, amplify with relatively the same reaction kinetics in the polymerase chain reaction as the wild-type target DNA (Porcher et al., 1992; Thiery et al., 1995).

The PCR reaction mixture was then put on a PCR Select III spin column (5 Prime --> 3 Prime, Inc.) in order to remove the excess primers and dNTPs still present in the reaction. The eluent was then ethanol precipitated overnight at -20 °C in the presence of 3M sodium acetate (pH 5.2) and glycogen. The DNA was pelleted by centrifugation, washed with 70% ethanol, and allowed to air dry. The purified PCR product was resuspended in deionized water and was then cloned using the TA Cloning Kit (Invitrogen). The PCR product (ITS-MUT) was ligated into the plasmid pCR™2.1.

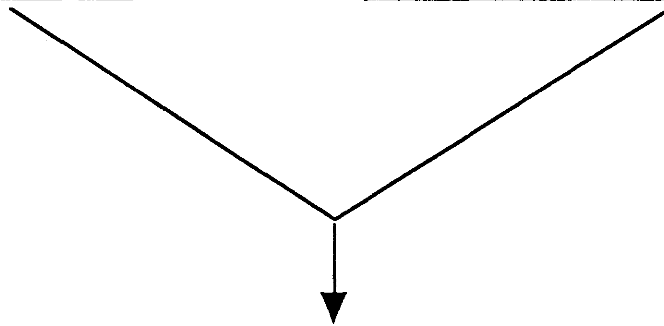
Figure 2. The nucleotide sequence of the original PER-ITS primer and the modified ITS-MUT primer are shown in bold type and underlined. The modified ITS-MUT primer contained the entire sequence of the original primer as well as an additional 10 bases located upstream within the ITS region. During amplification, the modified primer ITS-MUT (sequence shown at the bottom) annealed to the target DNA and looped out the 13 bases which are shown between the two bold underlined regions of sequence.

ORIGINAL PRIMER (PER-ITS)

AACCAGCGGTCTCGCTCTCTCTTGCT**CTTTTGTTAGAGAGTTGCGAGATG**GGA

MODIFIED PRIMER (ITS-MUT)

AAC**CAGCGGTCTC**GCTCTCTCTTGCT**CTTTTGTTAGAGAGTTGCGAGATG**GGA



CAGCGGTCTCTTTTGTTAGAGAGTTGCGAGATG

Competent *E. coli* INVαF′ cells were transformed with the plasmid. Plated colonies which did not express blue color in the presence of X-gal were selected for analysis (Sambrook et al., 1989).

Plasmid DNA was isolated using a PERFECTprep™ kit (5 Prime--> 3 Prime, Inc.) according to the manufacturer's recommendations. Briefly, cultures of the transformed *E. coli* cells were grown overnight in Luria broth at 37 °C. The cells were pelleted by centrifugation. Solution I (buffered RNase A solution) was added and the cells were resuspended by vortexing. In order to lyse the cells, Solution II (alkaline lysis solution) was added, and the tube was mixed by inversion. Solution III (potassium acetate solution) was added to neutralize the lysate. The cell lysate was centrifuged at 13000 x *g*. The resultant supernatant was applied to a spin column. DNA binding matrix (450 µl) was added to the spin column. After centrifugation for 30 seconds, the filtrate was decanted from the collection tube. DNA was precipitated by the addition of 400 µl of Purification Solution diluted with ethanol. After centrifugation, the filtrate was again decanted. TE which had been warmed to 65 °C was added to the spin column. Eluted plasmid DNA was collected via centrifugation in a fresh collection tube.

In order to ensure the presence of the deletion, the ITS-MUT plasmid was sequenced by the Sanger dideoxy chain termination method (Sanger et al., 1977). M13 universal forward and reverse primers were used. These primers were labeled with the LiCor IRD40 fluorescent dye. The ThermoSequenase kit (Amersham) was used for the sequencing reactions. The reactions were run on a 4% acrylamide gel, and the sequence was detected using the LiCor DNA 4000L automated sequencer.

The ITS-MUT plasmid DNA was quantitated using a Hoefer DyNA Quant 200 Fluorometer (Pharmacia Biotech Inc.). Finally, the ITS-MUT plasmid was digested with *Xba*I for 2 hours at 37 °C. *Xba*I does not cut within the 1197 base pair PCR product. The restriction endonuclease was inactivated by incubation at 65 °C for 20 minutes.

Digested plasmid DNA was stored at 4 °C until its subsequent use in all QCPCR reactions.

Construction of the control target plasmid

PCR was performed on *P. marinus* genomic DNA with the primers PER-18S and ITS-25 (primer D from Goggin, 1994) as described above for PCR with PER-18S and PER-ITS. The annealing temperature, however, was 55 °C. The resultant PCR product was cloned into a plasmid via the methods described above for the ITS-MUT competitor plasmid. The plasmid DNA was isolated, quantitated, and digested with *Xba*I as described for the ITS-MUT competitor plasmid. This cloned PCR product (ITS25) was used as a control target plasmid.

QCPCR

The QCPCR assay involved a two-phase approach. Titrations using a broad range of competitor dilutions are performed to obtain a rough estimate of the amount of DNA and then a second titration over a narrower range are performed for precise quantitation based on the methods of Gilliland et al. (1990b). The primer PER-18S was labeled on its 5' end with the fluorescent dye IRD41 (LiCor, Inc.). The first phase PCR included 23 µl of master mix containing reaction buffer (300 mM Tris-HCl pH 8.5, 75 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$), 3.75 pmoles of each primer (labeled PER-18S and unlabeled PER-ITS), 200 µM each of dATP, dCTP, dGTP, and dTTP, 400 ng/µl BSA, and 1 unit of Ampli*Taq* DNA polymerase (Perkin Elmer). A constant amount (1 µl) of target DNA of unknown concentration was added to each of a series of reactions containing the master mix and 1 µl of a known dilution of the competitor (5 pg/µl, 500 fg/µl, 50 fg/µl, 5 fg/µl, 0.5 fg/µl, or 0.05 fg/µl). Temperature cycling was carried out as described above.

The first phase of QCPCR spanned 6 orders of magnitude and often produced a sigmoidal curve when the log of the signal intensity ratio (target/competitor) was plotted against the log of the concentration of the competitor. Since quantitation should be done on the linear region of the fitted curve, the first phase QCPCR results were only used for estimating the dilution of the sample. Samples were diluted in TE to a concentration within the linear range of the QCPCR second phase reactions. The second phase narrow range PCR included 23 µl of master mix containing reaction buffer (Expand High Fidelity buffer with 15 mM $MgCl_2$ from Boehringer Mannheim Corporation), 3.75 pmoles of each primer (labeled PER-18S and unlabeled PER-ITS), 200 µM each of dATP, dCTP, dGTP, and dTTP, 400 ng/µl BSA and 2.6 units of the Expand High Fidelity DNA polymerase (cocktail of *Taq* polymerase and *Pwo* polymerase, Boehringer Mannheim Corporation). This enzyme mixture offered a greater sensitivity due to the presence of both *Taq* polymerase and *Pwo* polymerase. *Pwo* DNA polymerase has a 3' to 5' exonuclease activity which allowed for increased fidelity and overall the cocktail produced higher PCR yields. Oysters which were diagnosed with negative or rare infections were often not quantifiable by the QCPCR assay using the Ampli*Taq* DNA polymerase; however, the Expand High Fidelity system offered greater sensitivity and allowed quantitation of at least six of these previously undetected infections in the oyster samples. A constant amount (1 µl) of the diluted target DNA was added to each of a series of reactions containing the master mix and 1 µl of a known dilution of the competitor (1 fg/µl, 0.5 fg/µl, 0.1 fg/µl, 0.05 fg/µl, 0.01 fg/µl, or 0.005 fg/µl). Temperature cycling was as described above.

The PCR products of the competitor DNA and the target DNA were separated and visualized by acrylamide gel electrophoresis with a LiCor 4000L automated sequencer. A 4% acrylamide gel (12.6 g urea, 7.2 ml 5x TBE, 2.4 ml Long Ranger gel solution (FMC BioProducts), 220 µl 10% ammonium persulfate, 22 µl TEMED in a total volume of

30 ml) was poured between 25 cm glass plates 1 day prior to running. After PCR, reactions were diluted 1:3 in stop buffer/loading dye (95% formamide, 10 mM EDTA, 0.1% Bromophenol Blue, pH 9) and denatured at 92 °C for 2 minutes prior to loading. Approximately 0.5 µl of the diluted reaction was loaded onto the acrylamide gel. The automated sequencer was programmed to run at 2000 volts, 25 mA, 45 Watts, and at 45 °C. The motor speed for the laser was set to 2 which corresponds to 5.4 cm/second. After focusing, the signal gain was changed to 25 and the signal offset was changed to 102 to minimize the background. A second loading on the gel was done after the primer front moved past the detection laser. A total of 10 frames of data were collected by the LiCor BaseImagIR Data Collection software.

Amplification efficiency was determined for Ampli*Taq* DNA polymerase and the Expand™ High Fidelity PCR system individually. Plasmid target and plasmid competitor were used initially to determine amplification efficiency. Then, *P. marinus* genomic DNA isolated from cultured cells was used as the target to determine the amplification efficiency of the genomic DNA with the plasmid competitor. Reproducibility of the assay was assessed by replicate QCPCR with plasmid target and competitor and then with genomic DNA and plasmid competitor. Reproducibility was determined for Ampli*Taq* DNA polymerase and the Expand™ High Fidelity PCR system individually.

Oyster Collection

Oysters were collected from oyster bars at two sites in the James River, Virginia. Twenty five of these oysters were used in the final QCPCR analysis. Fourteen oysters were collected from Wreck Shoal in October 1996 or January 1997 and eleven oysters were collected from Point of Shoals in October or December 1996. Wreck Shoal has historically been a site of *P. marinus* infections of heavy intensity. Each oyster was

analyzed for the presence of *P. marinus* infection by QCPCR with hemolymph and QCPCR with gill and mantle tissue as well as by Ray's FTM method, FTM hemolymph assay, and whole oyster body burden RFTM analysis.

Perkinsus marinus RFTM Diagnosis--Hemolymph

Oyster shells were notched and hemolymph samples were taken using a 23 gauge needle and a 3 cc syringe. Approximately 0.6 ml of hemolymph was removed from each oyster and dispensed in equal volumes into two microcentrifuge tubes. One hemolymph aliquot was used for DNA isolation and the other was analyzed using the RFTM method of Gauthier and Fisher (1990). One ml of RFTM with 500 units of penicillin/streptomycin was added to the hemolymph. These tubes were incubated in the dark for at least 7 days. After incubation, the samples were centrifuged to pellet the cells, and the supernatant was removed. The pellet was resuspended in 1.0 ml of a 2M NaOH solution and incubated at room temperature for 30 minutes. After the samples were washed two times in distilled water, they were resuspended in 1.0 ml distilled water and then stained in 50 µl of a 1:6 dilution of Lugol's iodine stain. Three replicates of 100 µl each were aspirated onto 0.22 µm filter paper and the stained *P. marinus* cells were counted by light microscopy at 50x magnification. Samples containing large numbers of cells were diluted and counts were performed on the diluted samples. The entire sample volume was counted when less than 10 cells were present in 100 µl aliquots.

Perkinsus marinus RFTM Diagnosis--Standard

Oysters were shucked and pieces of gill, mantle, and rectal tissues approximately 0.25 g in size were dissected and incubated in 10 ml RFTM and penicillin/streptomycin (500 units/ml) for 7 days. After incubation, the tissues were removed, minced with a

razor blade, and stained with Lugol's iodine. Samples were examined under a light microscope for *P. marinus* infections according to the methods of Ray (1952).

Perkinsus marinus RFTM Diagnosis--Body Burden

After tissue pieces had been removed from the oyster for the standard RFTM assay and for the QCPCR assay, the remaining oyster tissue was minced with a razor blade. Minced tissue was weighed and added to a tube containing 20 ml of RFTM and penicillin/streptomycin (500 units/ml RFTM). The whole tissue assay was performed based on the procedure of Fisher and Oliver (1996). Samples were incubated in the dark for 7-10 days. After incubation, the samples were centrifuged to pellet the cells. The supernatant was removed, and the pellet was resuspended in 10 ml of 2M NaOH per gram of tissue weight. Then the samples were incubated at 60 °C for 1 hour. The digested samples were again centrifuged, and the supernatant was removed. Two washes of 20 ml distilled water were used to remove residual NaOH. The resulting pellet was resuspended in 1 ml of Lugol's (1:6) iodine solution. Three replicates of 100 µl each were aspirated onto 0.22 µm filter paper. Stained *P. marinus* cells were counted using a light microscope at 50x magnification. When less than 20 cells were present in the replicates, the entire sample volume was counted. If more than 300 cells were present in each replicate, dilutions were made and counts were performed on the diluted aliquots.

Isolation of DNA from hemolymph

A 0.3 ml aliquot of hemolymph from each oyster was used for isolation of DNA. Hemolymph samples were processed according to the methods of Stokes et al. (1995). Briefly, cells in hemolymph samples were washed with TE buffer (10 mM Tris, pH 8.0, 1.0 mM EDTA) and resuspended in lysing solution (50 mM Tris, pH 8.0, 100 mM EDTA, 1% Sarkosyl, 0.5 mg/ml proteinase K). After incubation overnight at 55 °C on a

rotator, the samples were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and then with an equal volume of chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated by the addition of 2.5 volumes of ethanol and 0.04 volumes 5 M NaCl. Samples were further purified by the ethidium bromide/high salt extraction procedure described above (Stemmer, 1991). DNA was resuspended in TE and stored at 4 °C. Before use in QCPCR, the DNA was digested with *Xba*I as described above.

Isolation of DNA from gill and mantle samples and rectum samples

Genomic DNA was isolated from the samples of oyster gill and mantle tissue using a modification of the method by Hill et al. (1991). The 0.25 g tissue samples were homogenized with disposable grinders in 0.25 ml of TE buffer in microcentrifuge tubes. The cells were pelleted by centrifugation, washed in TE buffer, and incubated in 25 µl of 5.9 M guanidine thiocyanate at 60° C for 90 minutes to lyse the cells. Lysates were diluted with dH_2O to achieve a final guanidine thiocyanate concentration of 0.3 M and then extracted twice with equal volumes of chloroform:isoamyl alcohol (24:1). DNA was precipitated at -20° C overnight with 0.6 volume isopropanol and 0.1 volume 3M NaOAc (pH 5.2). The resuspended DNA was further purified by the ethidium bromide/high salt extraction procedure as described above (Stemmer, 1991). Finally, the nucleic acids were resuspended in TE buffer and stored at 4 °C. Before use in QCPCR reactions, the DNA was digested with *Xba*I as described above.

Rectum samples (approximately 1/3 of the rectum) were put into microcentrifuge tubes containing 0.25 ml of TE buffer. DNA was isolated from rectum samples as for the gill and mantle samples.

Concentration of DNA

All DNA isolated from hemolymph samples and gill and mantle samples was reprecipitated before use in PCR reactions in order to increase the DNA concentration. Following the initial isolation, the volume of DNA resuspended in TE buffer was adjusted to 250 µl with dH_2O, and 140 µl of 7.5 M NH_4OAc, 1 ml 95% ethanol, and 10 µg of glycogen were added. DNA was precipitated overnight at -20 °C and pelleted by centrifugation. The concentrated DNA was resuspended in TE and stored at 4 °C. The recovery of DNA after ammonium acetate precipitation was found to be approximately 82% for the oyster tissue DNA samples by quantitation using the Hoefer DyNA Quant 200 Fluorometer (Pharmacia Biotech Inc.) before and after reprecipitation.

Standard curve preparation

Cultured *P. marinus* cells were enumerated using trypan blue exclusion staining. Briefly, equal volumes of cell culture and trypan blue were mixed and 25 µl of the mix were put onto a hemocytometer. Cells were counted using a light microscope at 50x magnification. Duplicate counts were made and cell viability was assessed. Dilutions of the culture were made in 0.22 µm filtered York River water.

Oysters which were suspected to be uninfected were obtained in June 1997 from Deep Water Shoals in the James River. At least 1 ml of hemolymph was taken from each oyster with a 23 gauge needle and divided into 0.3 ml samples. Oysters were shucked, and the gill and mantle tissue from each oyster was cut into 0.25 g samples. The hemolymph and gill and mantle tissues were frozen by immersion in liquid nitrogen and stored at -80 °C.

DNA was isolated from hemolymph samples and gill and mantle samples as described above. From each oyster, a hemolymph sample and a gill and mantle sample were screened for *P. marinus* infection using the PCR assay described above. No competitor

was added to these reactions and PCR was performed using the High Fidelity polymerase protocol as described above.

After obtaining negative results, the hemolymph samples and the gill and mantle samples were spiked with *P. marinus* cultured cells (10^2-10^6 cells) in triplicate. DNA was isolated from the spiked hemolymph samples and gill and mantle samples as described above. After digestion with *Xba*I, DNA was subjected to QCPCR as described above. Standard curves were constructed to relate the number of cultured *P. marinus* cells to the amount of DNA as determined by QCPCR analysis.

York River samples

Water samples were taken from the ferry pier in the lower York River at the Virginia Institute of Marine Science in Gloucester Point, Virginia on September 26 and October 13, 1997. One liter water samples were collected at a depth of 1 m. Water samples were filtered through a 35 µm nylon sieve to remove large particulate matter, and equal volumes of these samples were put into four 250 ml conical centrifuge bottles. The samples were centrifuged to pellet the particles, and the supernatants were removed by aspiration. The four resultant pellets were pooled and resuspended in 0.22 µm filtered York River water. Samples were stored at 4 °C until use.

DNA was isolated from the water samples according to the microwave prep method described above (Goodwin and Lee, 1993). DNA was isolated from one water sample from the September 26, 1997 sampling date and from three replicate water samples from the October 13, 1997 sampling date. This protocol was again modified to include the CTAB extractions in order to eliminate inhibitors of *Taq* DNA polymerase, which are commonly found in environmental samples (Wilson, 1994). The DNA samples were digested with *Xba*I and submitted to QCPCR analyses as described above. Before

loading, the PCR reactions were diluted 1:2 with stop buffer/loading dye. Approximately 1 µl of the diluted reaction was loaded onto the acrylamide gel.

Three additional water samples taken on October 13, 1997 were filtered and centrifuged as described above. The pellets, however, were pooled and resuspended in a tube containing 20 ml of RFTM and penicillin/streptomycin (500 units/ml RFTM). These samples were incubated in the dark for 7-10 days. This method was similar to that described for the whole oyster body burden assay described by Fisher and Oliver (1996). After incubation, the samples were centrifuged to pellet the cells. The supernatant was removed, the pellet was resuspended in 25 ml of 2M NaOH, and the samples were incubated at 60 °C for 1 hour. The digested samples were again centrifuged, and the supernatant was removed. Two washes of 20 ml distilled water were used to remove residual NaOH. The resulting pellet was resuspended in 1 ml of Lugol's (1:6) iodine solution. Counts of the entire sample were made using the light microscope at 50x magnification.

Data Analysis

The software package GeneImagIR (RFLPscan, Scanalytics) was utilized to calculate the peak areas under the Gaussian curves (integrated density of the bands) fitted to both the target and competitor PCR product bands. The signal intensity or integrated density value represents the peak area which is a measure for the relative fluorescence of the product band as well as the time a specific fragment takes to migrate past the scanning window of the laser beam. This peak area is proportional to the molar amount of the DNA fragment when fragments of similar length are compared (Hahn et al., 1995). The logarithm of the ratio of the integrated densities of the product bands (target/competitor) was plotted against the logarithm of the amount of competitor initially added to the reaction (Cross, 1995). Linear regressions were performed on the data, including all

replicates (Zar, 1984), in order to assess the relationship of the variables. The regression curve equation was given in the form y = mx + b where m represents the slope of the line. The equivalence point was determined by solving the regression equation for the curve when the ratio of signal intensity of the target/competitor is 1 (y=0) (Piatak et al., 1993; Zimmermann and Mannhalter, 1996).

The amount of DNA in the target sample as determined by the equivalence point was corrected for dilution, volume of DNA prep used in the PCR, and reprecipitation recovery. In addition, a genomic correction factor was determined for each enzyme used in the QCPCR assay. The genomic correction factor was used to correct for the difference in the number of the specific sequence in equivalent amounts of target and genomic DNA. The correction factor was determined by correcting the equivalence point for each of the genomic target concentrations within the range of quantitation. For Ampli*Taq* DNA polymerase, the correction factor was determined from the series of reactions with 1 µl of the 10 ng/µl, 1 ng/µl, 500 pg/µl, 50 pg/µl, 20 pg/µl, and 10 pg/µl *P. marinus* genomic target DNA. For the Expand™ High Fidelity PCR system, the genomic correction factor was determined from QCPCR with 1 µl of the *P. marinus* genomic target DNA at concentrations of 5 fg/µl, 1 fg/µl, and 0.5 fg/µl . Only the narrow range (2nd phase) QCPCR was used for the Expand™ High Fidelity genomic correction factor since this enzyme was used only with this method. The correction factors determined from each concentration of genomic target DNA were averaged for each of the enzymes. For all oyster tissue samples, the Expand™ High Fidelity genomic correction factor was used to correct the estimated DNA concentration. Finally, the amount of DNA calculated by QCPCR was converted to cells per gram for gill and mantle samples or to cells per milliliter for hemolymph samples using the appropriate equation for the linear regression. The 95% confidence intervals were calculated for each standard curve linear regression in order to estimate the standard error of the slope of the line.

Individual data points on the hemolymph standard curve and the gill and mantle standard curve were determined identically to the gill and mantle or hemolymph samples.

Comparisons of the weight standardized parasite burden obtained from the whole tissue assay or the hemolymph assay, the intensity rank obtained from Ray's FTM assay, and the QCPCR diagnoses were made in pairs. Regression analyses were employed to illustrate any specific relationships between the assays. The significance of the relationship was determined by the p value of the ANOVA F statistic for each pair of assays.

RESULTS

PCR Sensitivity and Specificity

Perkinsus marinus specific primers PER-18S and PER-ITS amplified a 1210 base pair region of DNA from the small subunit ribosomal RNA gene and first internal transcribed spacer region (Figure 1). This region is present in the genome in multiple copies. Amplifying a sequence from this multi-copy region provided more copies of the template sequence for the annealing of the PCR primers. Thus, the sensitivity of the assay was increased. On an ethidium bromide stained agarose gel, the PCR product resulting from amplification with 100 fg of DNA isolated from *P. marinus* cultured cells was easily detected. Using the LiCor automated sequencer to detect fluorescently-labeled PCR products, as little as 0.005 fg of *P. marinus* DNA in a background of approximately 0.4 µg of *C. virginica* DNA was detected on a polyacrylamide gel. Furthermore, the LiCor automated DNA sequencer detected *P. marinus* PCR product from the DNA of 2 cultured cells in a background of approximately 5 mg of oyster tissue. Using 1 µl of unpurified PCR product in a second amplification step did not increase the sensitivity of the assay.

These primers did not have any significant homology to *Crassostrea virginica*, dinoflagellates, *Haplosporidium nelsoni*, or other *Perkinsus* species when compared to sequences from Genbank. Amplification with DNA isolated from cultured *Perkinsus-1* cells yielded the expected PCR product of 1210 bp. Furthermore, DNA isolated from geographic isolates of *P. marinus* cultured cells from Virginia, Connecticut, South

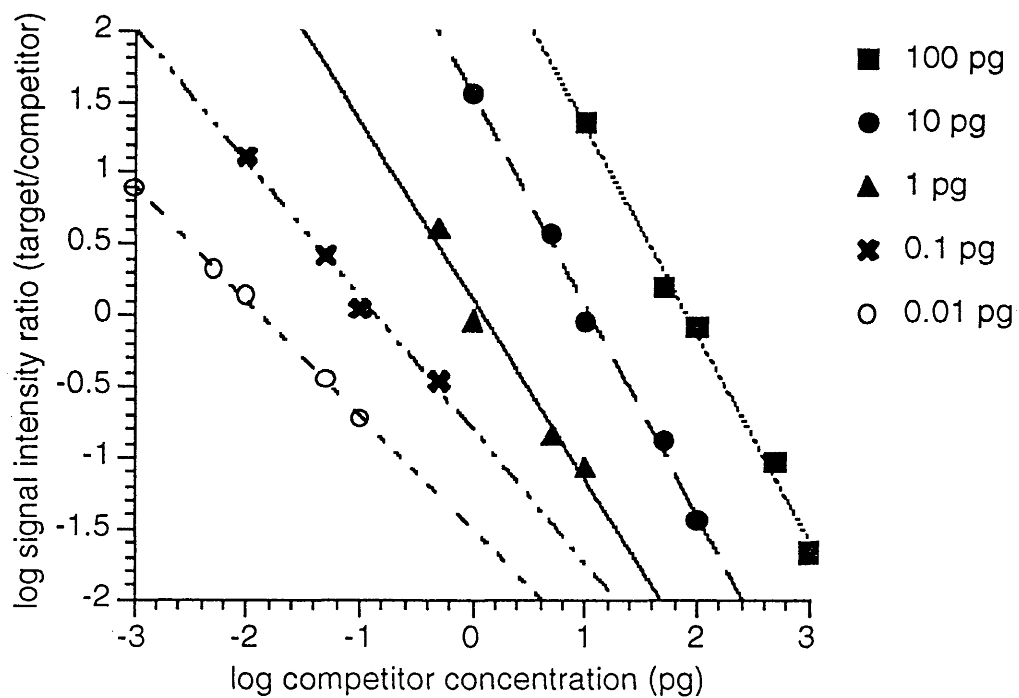
Carolina, Louisiana, and Texas amplified with the PER-18S and PER-ITS primers under the conditions described for this PCR assay.

DNA isolated from *Crassostrea virginica*, *Argopecten irradians*, *Amphidinium* sp., *Gymnodinium* sp., and *Peridinium* sp. yielded the appropriate PCR product when tested with "universal" actin gene primers. No PER-18S and PER-ITS PCR product was detectable on an ethidium bromide stained agarose gel after amplification with *Crassostrea virginica* DNA. In addition, no product band was detected by the LiCor automated DNA sequencer on an acrylamide gel after PCR with *C. virginica* DNA and the fluorescently-labeled *Perkinsus marinus* primer set. DNA isolated from *C. virginica* tissue spiked with cultured *P. marinus* cells yielded the appropriate PCR product. DNA isolated from the scallop *Argopecten irradians* and spiked with DNA from cultured *P. marinus* cells produced one PCR product band of 1210 bp. When DNA isolated from the dinoflagellates *Amphidinium* sp., *Gymnodinium* sp., and *Peridinium* sp. were tested, no product was detected after PCR with PER-18S and PER-ITS. More importantly, no product was detected after PCR with the PER-18S and PER-ITS primers and DNA isolated from cultured *Perkinsus atlanticus* cells.

Quantitative Competitive Polymerase Chain Reaction (QCPCR)

Series of reactions with plasmid target DNA concentrations ranging from 100 pg/µl to 10 fg/µl are depicted in Figure 3. Each of the six target concentrations is represented by an individual curve which corresponds to the series of dilutions of the competitor. The regression curves (log signal intensity ratio (target/competitor) vs. log concentration of competitor) show slopes ranging from -1.45 to -0.805. Quantitation was possible for this entire range of plasmid target concentrations. The equivalence points for each of the series of reactions were found to be 81.010 pg, 12.113 pg, 1.212 pg, 0.140 pg, and 0.0134 pg for 100 pg, 10 pg, 1 pg, 0.1 pg and 0.01 pg of target DNA respectively.

Figure 3. QCPCR with plasmid target DNA (ITS25) and plasmid competitor DNA (ITS-MUT) with Ampli*Taq* DNA polymerase. 100 pg, 10 pg, 1 pg, 0.1 pg, or 0.01 pg of the plasmid target DNA was used in each QCPCR series. The linear regression equations are given in the form $f(x) = mx + b$ where m is the slope of the linear curve. R^2 values for each of the regressions are given.



100 pg	$f(x) = -1.452182\ x + 2.77154$	$R^2 = 0.993$
10 pg	$f(x) = -1.472170\ x + 1.54049$	$R^2 = 0.996$
1 pg	$f(x) = -1.261984\ x + 0.105234$	$R^2 = 0.973$
0.1 pg	$f(x) = -0.939507\ x - 0.802079$	$R^2 = 0.990$
0.01 pg	$f(x) = -0.804703\ x - 1.50639$	$R^2 = 0.998$

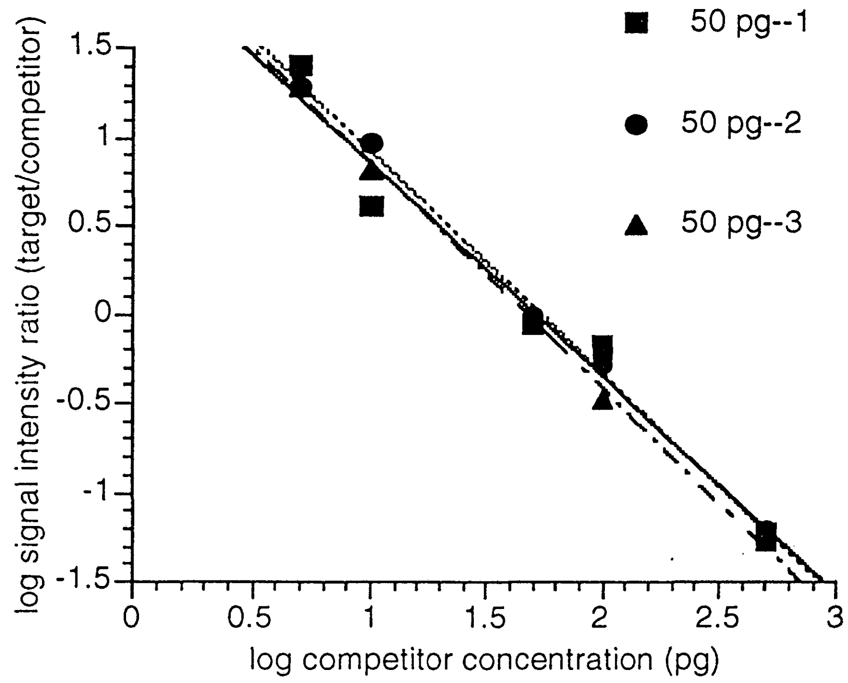
These values represent the initial amount of target DNA in the reaction before amplification. Triplicate QCPCRs were run for 2 concentrations of target plasmid DNA, 50 pg/µl and 0.05 pg/µl (Figure 4). All replicate series yielded slopes close to -1. For these replicates, the equivalence point was found at 51.059 ± 3.636 pg for the 50 pg series (Figure 4A) and at 0.056 ± 0.004 pg for the 0.05 pg series (Figure 4B).

QCPCRs with known concentrations of genomic target DNA ranging from 10 ng/µl to 10 pg/µl were run with the appropriate series of competitor plasmid DNA (Figure 5). Slopes of the fitted curves ranged from -0.842 to -1.39 indicating the nearly equivalent amplification efficiencies of the genomic target and plasmid competitor. Digestion of the DNA with *Xba*I made the amplification efficiencies of genomic and plasmid DNA more equivalent. For the series of reactions with 1 µl of the genomic target DNA concentrations of 10 ng/µl, 1000 pg/µl, 500 pg/µl, 100 pg/µl, 50 pg/µl, 20 pg/µl, and 10 pg/µl, the QCPCR assay yielded equivalence points at 0.269 ng, 26.122 pg, 13.032 pg, 2.523 pg, 1.067 pg, 0.449 pg, and 0.209 pg. Utilizing these calculated equivalence points, the genomic correction factor was determined to be 41.825 ± 4.460 for Ampli*Taq* DNA polymerase. This genomic correction factor was subsequently used to correct for differences in the number of target sequences in pg of genomic DNA versus pg of plasmid DNA. The reproducibility of the QCPCRs with genomic target DNA and plasmid competitor DNA is shown with three replicates for each of the two concentrations used (Figure 6). For 500 pg of genomic target DNA, the equivalence point was found at 9.234 ± 0.199 pg. For 50 pg of genomic target DNA, the equivalence point was found at 0.931± 0.042 pg. Using the genomic correction factor for the Ampli*Taq* DNA polymerase, the corrected equivalence points were found to be 386.2 pg and 38.9 pg for the 500 pg and 50 pg reactions respectively.

The amplification efficiencies of the plasmid target and plasmid competitor molecules were then tested for QCPCR with the High Fidelity system (Figure 7). Target

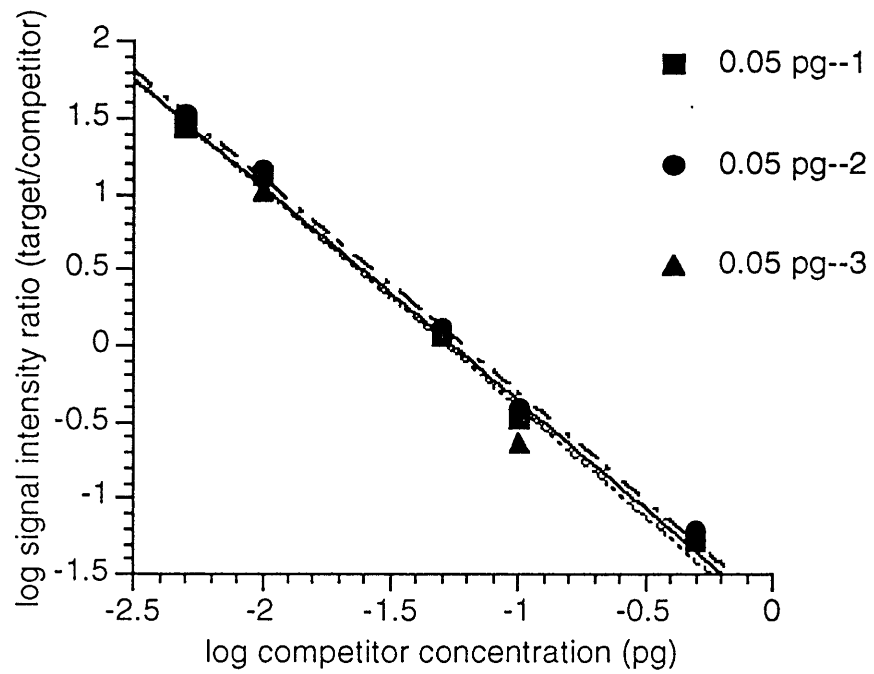
Figure 4. QCPCR with plasmid target DNA (ITS25) and competitor plasmid (ITS-MUT) with Ampli*Taq* DNA polymerase. Reproducibility was assessed with three replicate QCPCRs (designated 1, 2, and 3) for each target DNA concentration. The linear regression equations are given in the form f(x) = mx + b where m is the slope of the linear curve. R^2 values are given for each of the regressions. (A) 50 pg plasmid target DNA. (B) 0.05 pg plasmid target DNA.

A



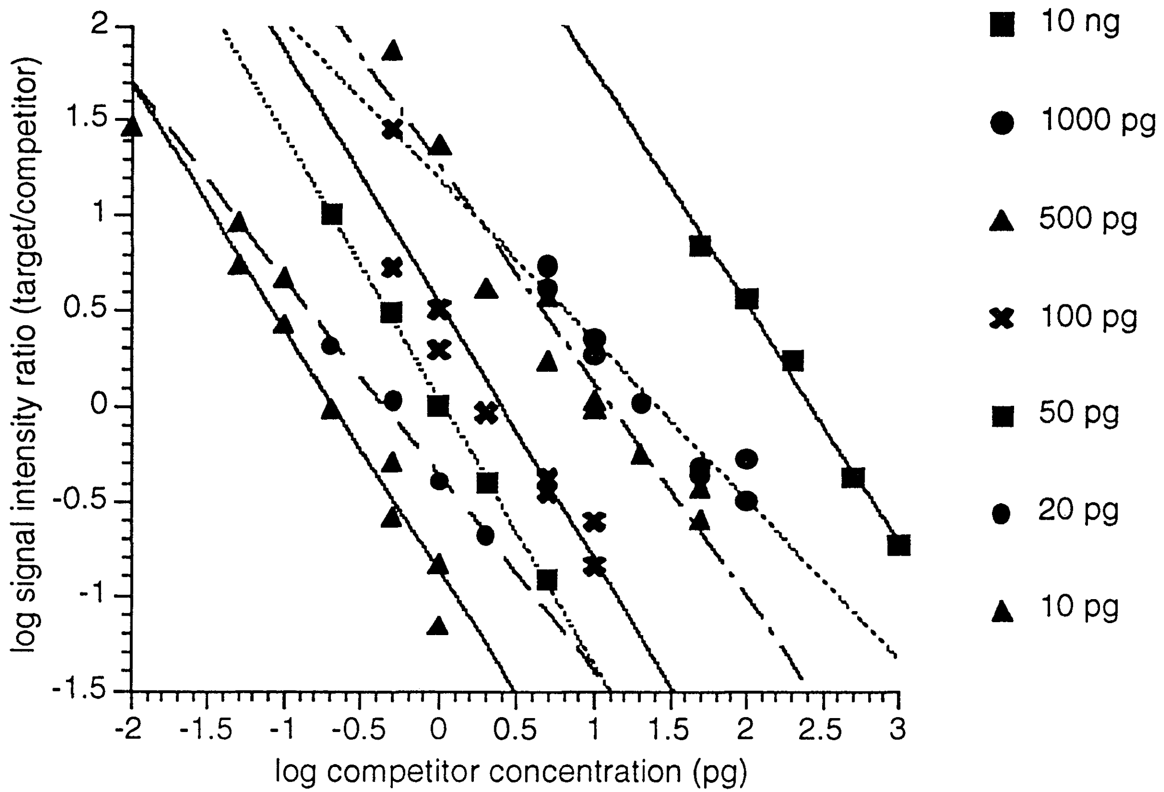
1	$f(x) = -1.20743\,x + 2.06416$	$R^2 = 0.967$
2	$f(x) = -1.25417\,x + 2.17828$	$R^2 = 0.998$
3	$f(x) = -1.27554\,x + 2.13593$	$R^2 = 0.998$

B



1	$f(x) = -1.40569\ x - 1.76739$	$R^2 = 0.995$
2	$f(x) = -1.40747\ x - 1.71364$	$R^2 = 0.996$
3	$f(x) = -1.43344\ x - 1.83791$	$R^2 = 0.986$

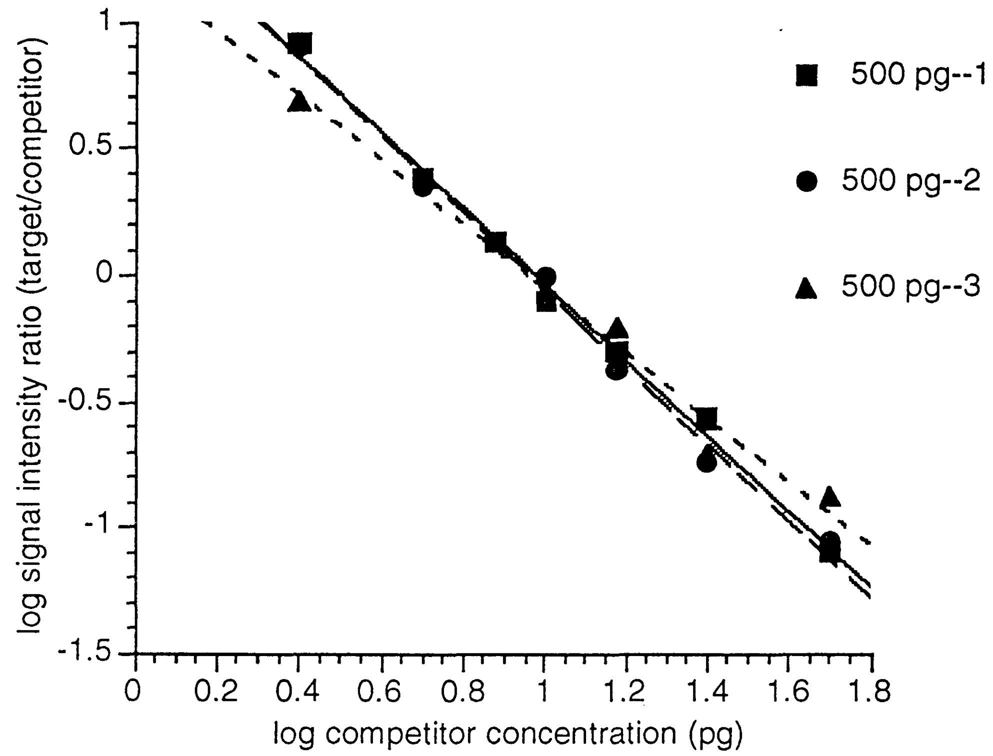
Figure 5. QCPCR with *P. marinus* genomic target DNA and plasmid competitor DNA with Ampli*Taq* DNA polymerase. 10 ng, 1 ng, 500 pg, 100 pg, 50 pg, 20 pg, or 10 pg of genomic target DNA was used in each of the QCPCR series. The linear regression equations are given in the form $f(x) = mx + b$ where m is the slope of the linear curve. R^2 values are given for each of the regressions.



10 ng	$f(x) = -1.23857\ x + 3.00780$	$R^2 = 0.991$
1000 pg	$f(x) = -0.841796\ x + 1.19260$	$R^2 = 0.940$
500 pg	$f(x) = -1.13590\ x + 1.26613$	$R^2 = 0.941$
100 pg	$f(x) = -1.34225\ x + 0.539875$	$R^2 = 0.898$
50 pg	$f(x) = -1.39283\ x + 0.0393233$	$R^2 = 0.999$
20 pg	$f(x) = -1.03067\ x - 0.358825$	$R^2 = 0.984$
10 pg	$f(x) = -1.28272\ x - 0.871190$	$R^2 = 0.957$

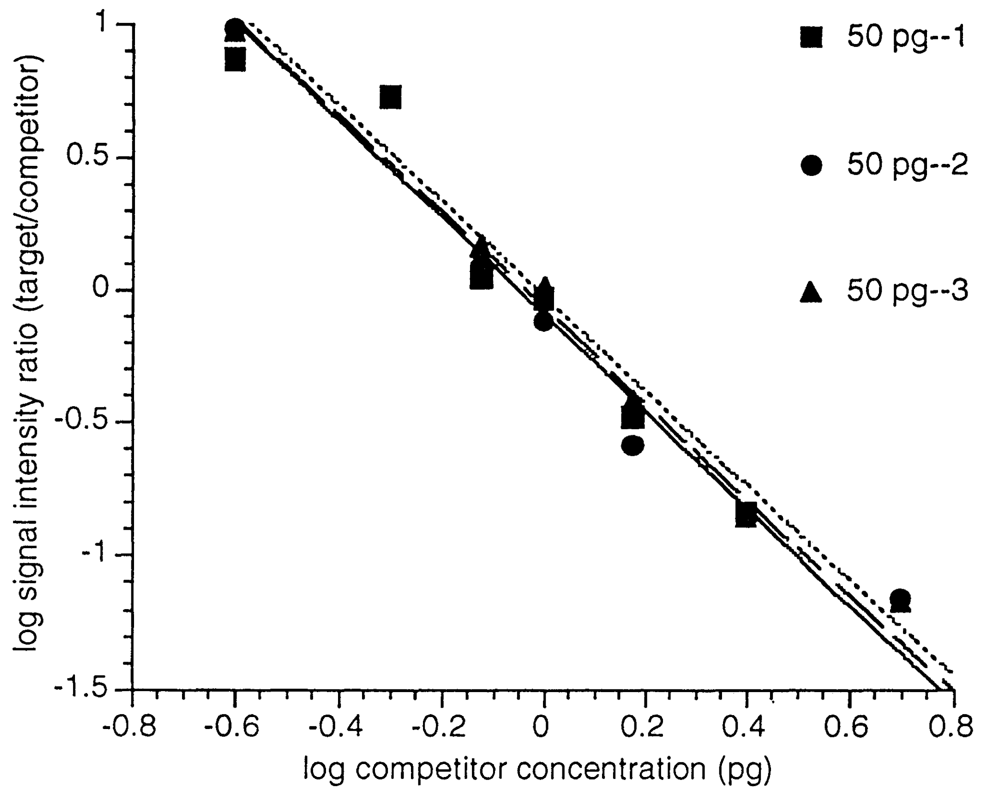
Figure 6. QCPCR with *P. marinus* genomic target DNA and competitor plasmid (ITS-MUT) with Ampli*Taq* DNA polymerase. Reproducibility was assessed with three replicate QCPCRs (designated 1, 2, and 3) for each target DNA concentration. The linear regression equations are given in the form f(x) = mx + b where m is the slope of the linear curve. R^2 values are given for each of the regressions. (A) 500 pg genomic target DNA. (B) 50 pg genomic target DNA.

A



1	$f(x) = -1.50002\ x + 1.46458$	$R^2 = 0.995$
2	$f(x) = -1.52637\ x + 1.46747$	$R^2 = 0.992$
3	$f(x) = -1.27321\ x + 1.22078$	$R^2 = 0.980$

B

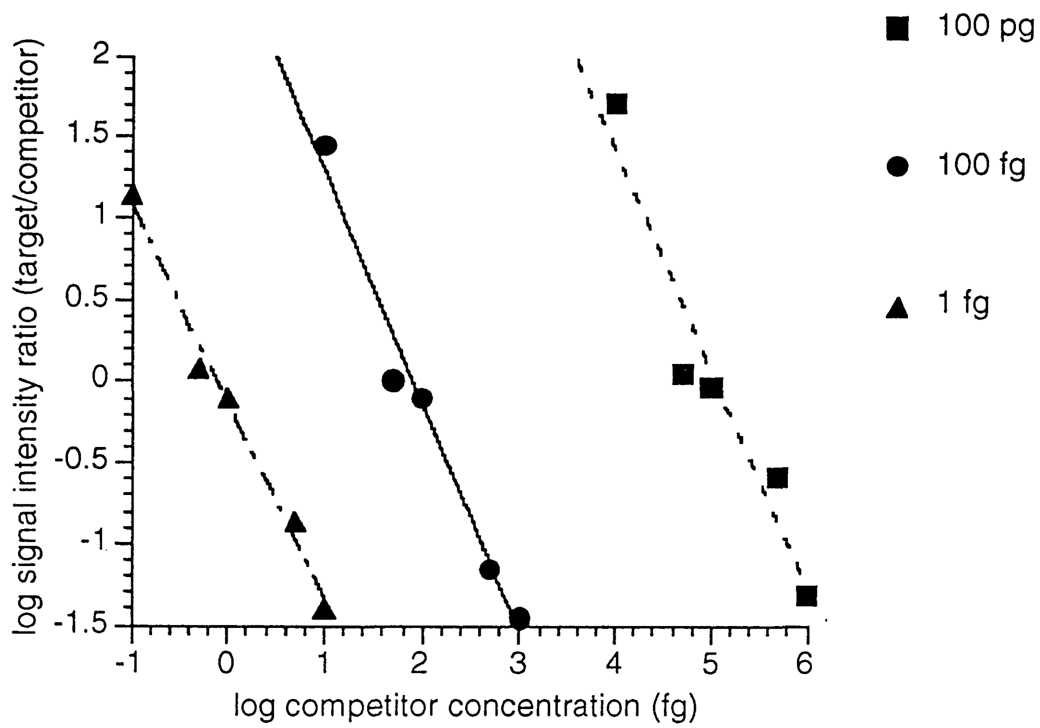


1 $f(x) = -1.82438\,x - 0.0883574$ $R^2 = 0.949$

2 $f(x) = -1.79429\,x - 0.0643867$ $R^2 = 0.962$

3 $f(x) = -1.78545\,x - 0.0174909$ $R^2 = 0.977$

Figure 7. QCPCR with plasmid target DNA (ITS25) and plasmid competitor DNA (ITS-MUT) with Expand^{TM} High Fidelity PCR System DNA polymerase. 100 pg, 100 fg, or 1 fg of plasmid target DNA was used in each of the QCPCR series. The linear regression equations are given in the form f(x) = mx + b where m is the slope of the linear curve. R^2 values for each of the regressions are given.



100 pg	$f(x) = -1.35075\ x + 6.82418$	$R^2 = 0.929$
100 fg	$f(x) = -1.42086\ x + 2.70557$	$R^2 = 0.977$
1 fg	$f(x) = -1.20795\ x - 0.129458$	$R^2 = 0.988$

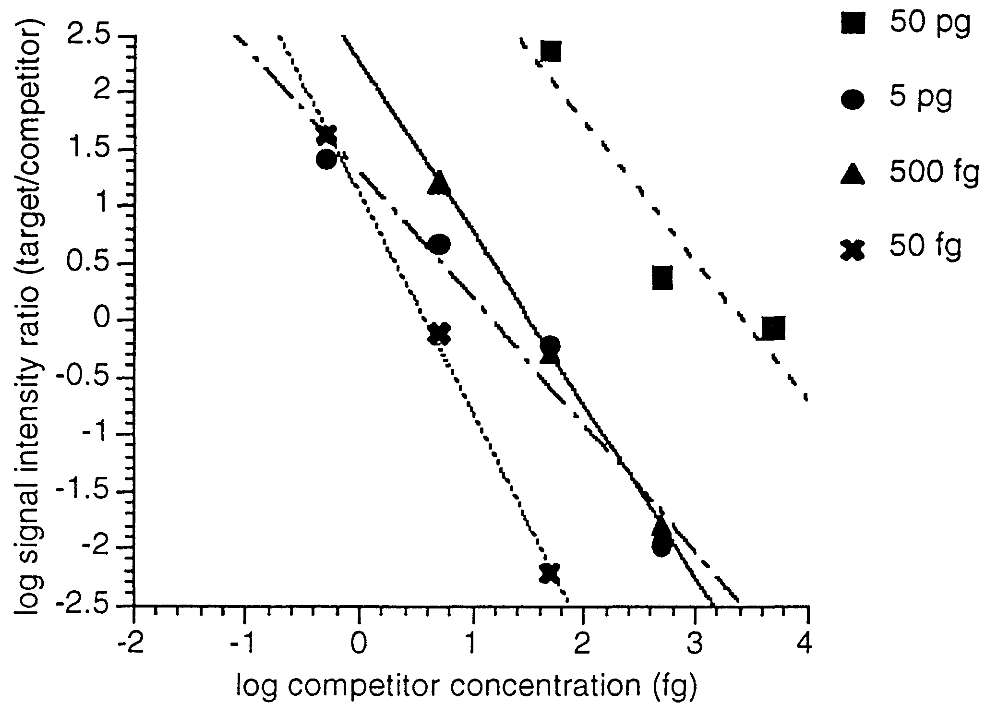
concentrations were 100 pg/μl, 100 fg/μl, and 1 fg/μl. The corresponding equivalence points were found to be 112.720 pg, 80.168 fg, and 0.782 fg. The slopes of the log-log plots, which ranged from -1.21 to -1.42, indicated that the target and competitor plasmids were amplifying with relatively equal efficiencies. After the plasmid target DNA was tested, genomic DNA isolated from cultured *P. marinus* cells was used in QCPCR reactions with the competitor plasmid (Figure 8). Due to the equivalent amplification efficiencies depicted, quantitation was possible for 50 pg to 0.5 fg of target DNA. In Figure 8B, the QCPCR second phase reactions with 5 fg, 1 fg, and 0.05 fg of *P. marinus* genomic DNA resulted in the determination of equivalence points at 0.489 fg, 0.134 fg, and 0.119 fg respectively. The narrow range series of reactions allowed a genomic correction factor of 7.296 ± 3.015 to be calculated for the more specific, higher yielding High Fidelity system. Reproducibility for genomic target DNA and plasmid competitor DNA was assessed for the High Fidelity system with 1 pg and 5 fg of target DNA (Figure 9). Because these values fell at approximately the midpoint of the broad and narrow range competitor series after conversion using the genomic correction factor, these concentrations were used for reproducibility assessment. The equivalence point for 1000 fg of target DNA was found to be at 110.979 ± 29.785 fg while the equivalence point for 5 fg of target DNA was found to be at 0.340 ± 0.079 fg. Utilizing the genomic correction factor for the High Fidelity PCR System, the corrected values were 809.7 fg and 2.483 fg respectively.

Perkinsus marinus Infection Diagnosis

The *P. marinus* infection intensities of the twenty five oysters processed by QCPCR, Ray's FTM assay (Ray, 1952), whole oyster body burden (Fisher and Oliver, 1996), and FTM hemolymph assay (Gauthier and Fisher, 1990) are described below. The gel image

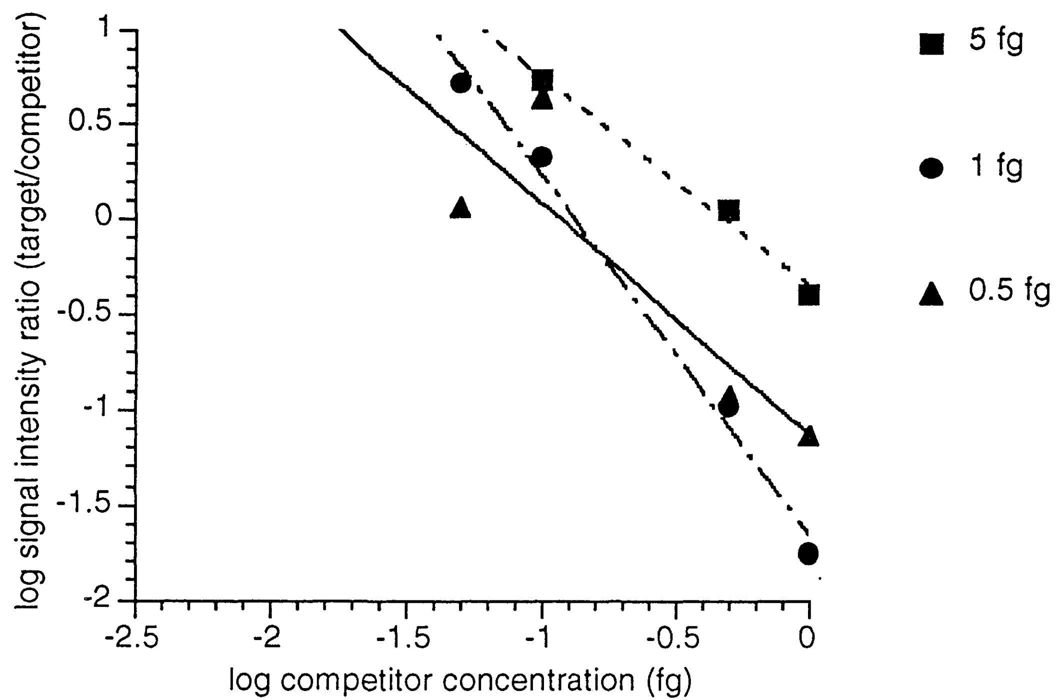
Figure 8. QCPCR with *P. marinus* genomic target DNA and plasmid competitor DNA with Expand™ High Fidelity PCR system DNA polymerase. The linear regression equations are given in the form f(x) = mx + b where m is the slope of the linear curve. R^2 values are given for each of the regressions. (A) 50 pg, 5 pg, 500 fg, or 50 fg of genomic target DNA was used in each of the QCPCR series. Plasmid competitor DNA concentrations were 5 pg/µl, 500 fg/µl, 50 fg/µl, 5 fg/µl, 0.5 fg/µl, and 0.05 fg/µl as described for the broad range QCPCR. (B) 5 fg, 1 fg, or 0.5 fg of genomic target DNA was used in each of the QCPCR series. Plasmid competitor DNA concentrations were 1 fg/µl, 0.5 fg/µl, 0.1 fg/µl, 0.05 fg/µl, 0.01 fg/µl, and 0.005 fg/µl as described for the narrow range QCPCR.

A



50 pg	$f(x) = -1.21648\ x + 4.17345$	$R^2 = 0.881$
5 pg	$f(x) = -1.10419\ x + 1.29469$	$R^2 = 0.957$
500 fg	$f(x) = -1.50373\ x + 2.26796$	$R^2 = 0.929$
50 fg	$f(x) = -1.92293\ x + 1.10838$	$R^2 = 0.997$

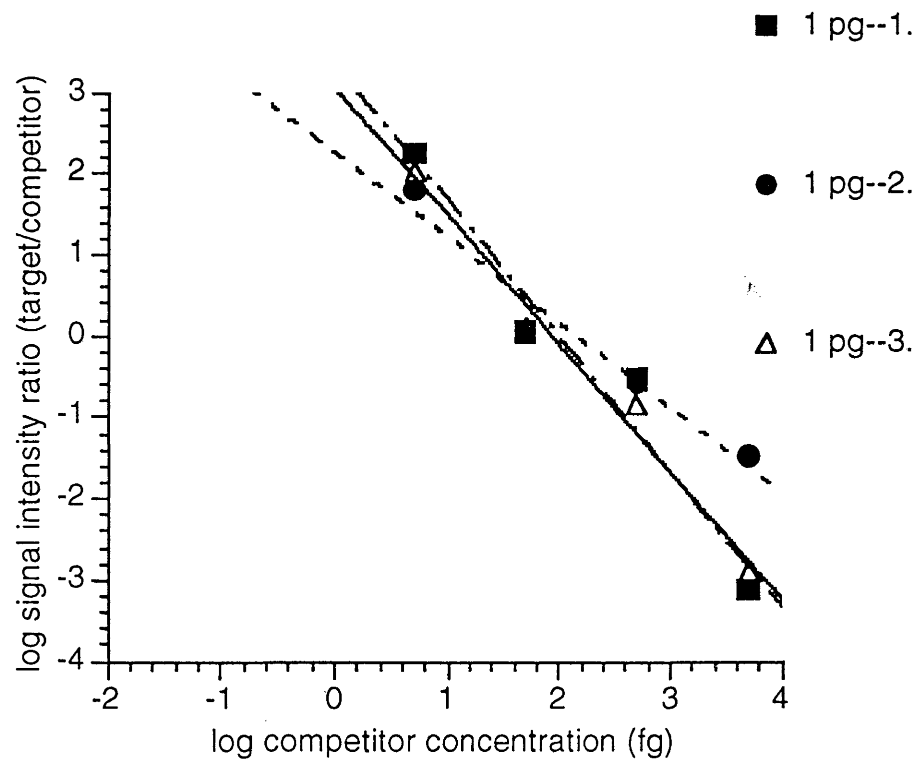
B



5 fg	$f(x) = -1.09917\ x - 0.343080$	$R^2 = 0.989$
1 fg	$f(x) = -1.89628\ x - 1.65426$	$R^2 = 0.990$
0.5 fg	$f(x) = -1.21875\ x - 1.12727$	$R^2 = 0.769$

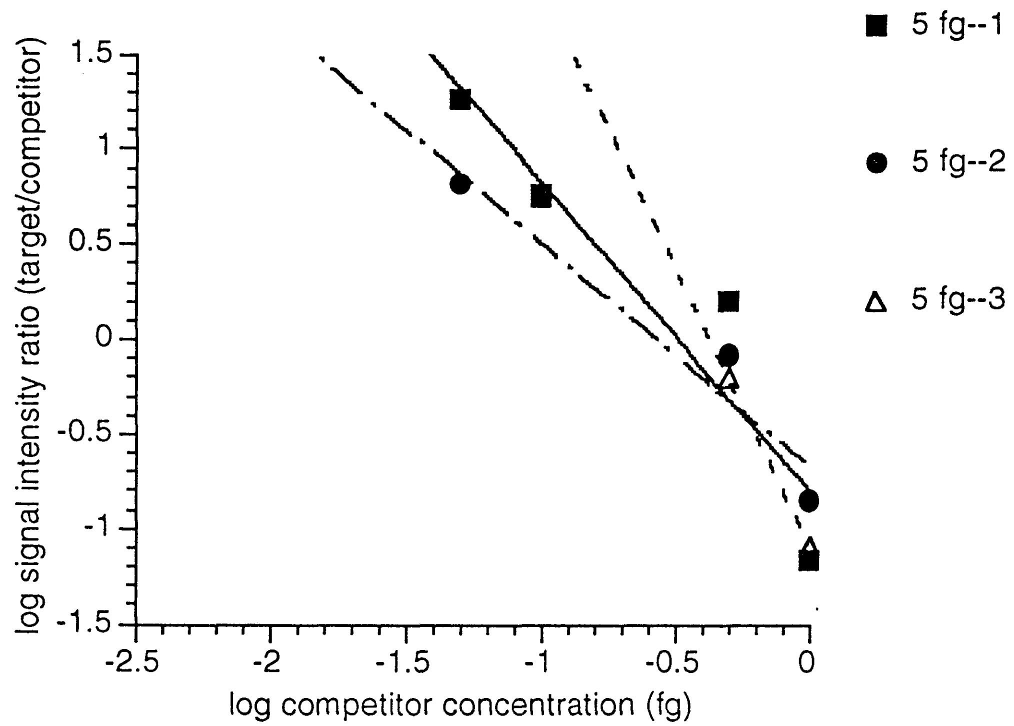
Figure 9. QCPCR with *P. marinus* genomic target DNA and competitor plasmid (ITS-MUT) with Expand™ High Fidelity PCR system DNA polymerase. Reproducibility was assessed with three replicate QCPCRs (designated 1, 2, and 3) for each target DNA concentration. Plasmid competitor DNA concentrations were 1 fg/µl, 0.5 fg/µl, 0.1 fg/µl, 0.05 fg/µl, 0.01 fg/µl, and 0.005 fg/µl for each QCPCR series. The linear regression equations are given in the form f(x) = mx + b where m is the slope of the linear curve. R^2 values are given for each of the regressions. (A) 1 pg genomic target DNA. (B) 5 fg genomic target DNA.

A



1	$f(x) = -1.66929\,x + 3.34425$	$R^2 = 0.952$
2	$f(x) = -1.04622\,x + 2.25979$	$R^2 = 0.954$
3	$f(x) = -1.56493\,x + 3.04078$	$R^2 = 0.982$

B



1	$f(x) = -1.62862\ x - 0.795978$	$R^2 = 0.877$
2	$f(x) = -1.18409\ x - 0.671827$	$R^2 = 0.936$
3	$f(x) = -2.93887\ x - 1.08979$	$R^2 = 1.000$

(Figure 10) depicts a representative gill and mantle sample (Oyster 18) second phase series of QCPCR reactions because only the second phase portion of the QCPCR assay was used to quantitate the amount of DNA in the sample. A plot of the log of the signal intensity ratio (target/competitor) versus the log of the concentration of the competitor for the same gill and mantle sample (Oyster 18) is shown in Figure 11. A linear regression of these data yielded a curve with equation $y= -0.271 - 1.347 x$. Solving this equation for $y=0$, the value of x at the equivalence point was determined. The amount of DNA in fg was calculated to be 0.63 fg from this graph. This value was corrected for reprecipitation recovery (0.82), dilution (200000) and enzyme genomic correction factor (7.296). Finally, the results were transformed to yield DNA per gram of tissue (x4). The resultant amount of *P. marinus* DNA was found to be 3015290.88 fg (or 3.02 µg) per gram of tissue for Oyster 18.

For rectal tissue samples, PCR inhibitors prevented consistent amplification in even the heaviest infections. PCR product bands were not detected after the first amplification in 9 of 25 oysters which were subsequently found to be positive for *Perkinsus marinus* by gill and mantle QCPCR, Ray's FTM assay, and the body burden assay.

Standard curves which related the amount of DNA (fg) to the number of cultured *Perkinsus marinus* cells in gill and mantle (Figure 12) and in hemolymph (Figure 13) are shown. The regression equation $y=0.273 + 1.215 x$ from the log-log graph was used to calculate the number of *P. marinus* cells per gram of gill and mantle tissue. Thus, for the gill and mantle sample from Oyster 18, there were 128,825 *P. marinus* cells per gram of oyster tissue. The 95% confidence intervals were determined in order to estimate the range of numbers of cells which could be calculated for the corresponding amount of DNA (3.02 µg). For the hemolymph samples, the regression equation $y=3.038 + 0.903 x$ from the log-log graph was used to calculate the number of *P. marinus* cells per milliliter of hemolymph.

Figure 10. LiCor automated sequencer acrylamide gel image of fluorescently-labeled PCR products. The image depicts the QCPCR with the Oyster 18 gill and mantle sample. The 1210 bp band is the target DNA product and the 1197 bp band is the competitor DNA product. Each lane represents a single QCPCR with a constant aliquot of genomic target DNA. In addition, one of a series of dilutions of the plasmid competitor DNA was added to each reaction: 1 fg, 0.5 fg, 0.1 fg, 0.05 fg, 0.01 fg, and 0.005 fg (lanes 1-6 respectively).

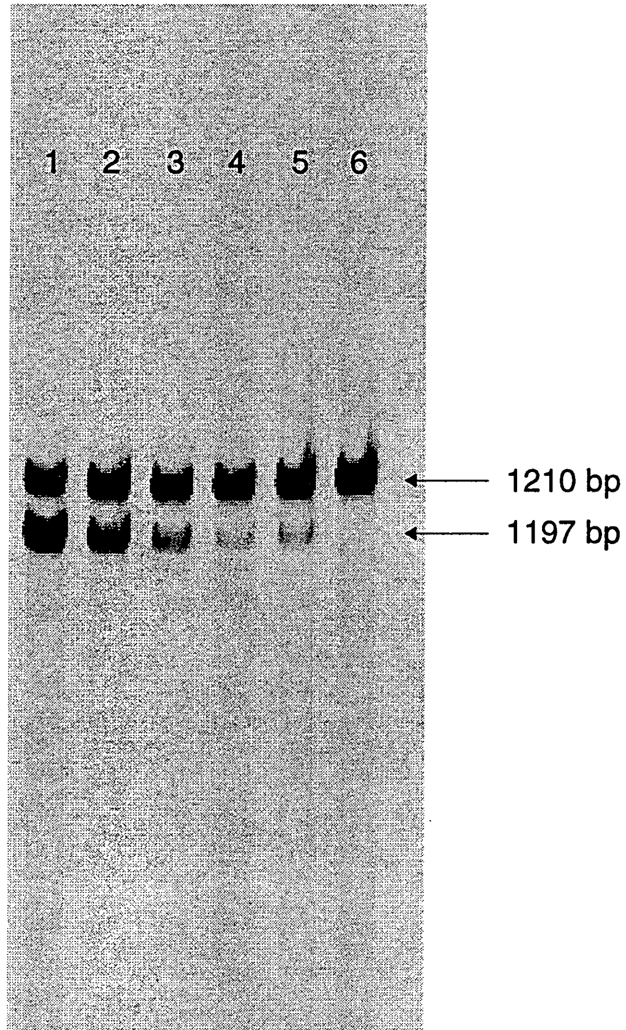
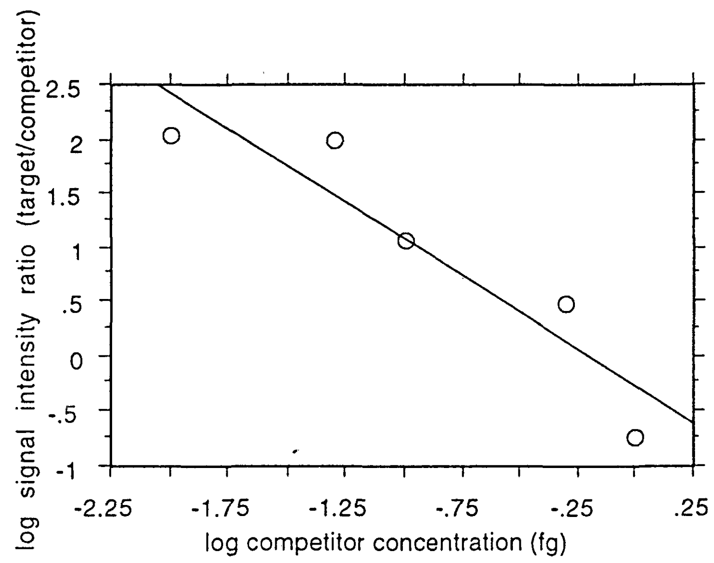
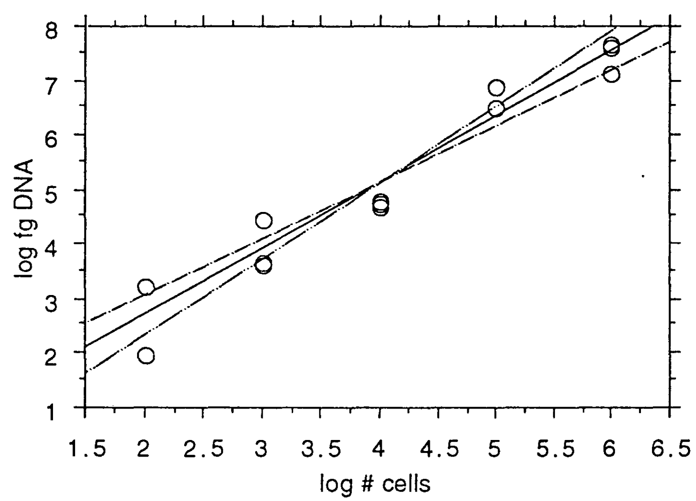

1 2 3 4 5 6
1210 bp
1197 bp

Figure 11. QCPCR with genomic target DNA isolated from Oyster 18 gill and mantle sample. A constant aliquot of the genomic DNA isolated from the Oyster 18 gill and mantle tissue and one of the series of dilutions of the plasmid competitor DNA (1 fg, 0.5 fg, 0.1 fg, 0.05 fg, 0.01 fg, and 0.005 fg) composed each of the reactions. The linear equation and the R^2 value is given for the regression.



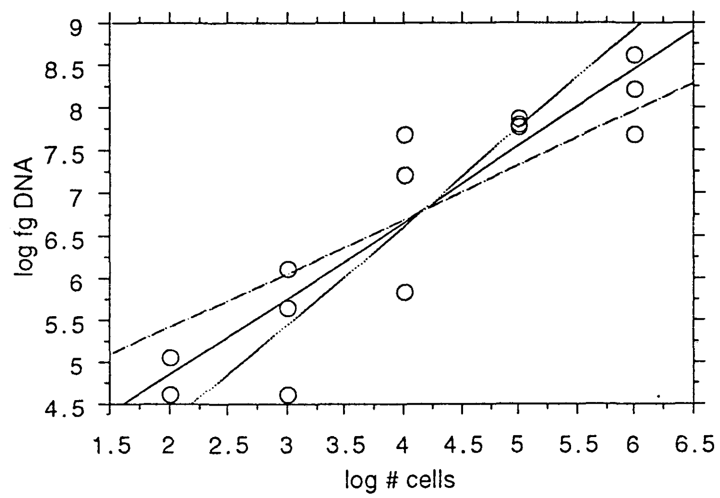
$f(x) = -1.347\ x - 0.271$ $R^2 = 0.863$

Figure 12. Gill and mantle tissue standard curve illustrates the relationship between the log of the amount of DNA (fg) determined by QCPCR and the log of the number of *Perkinsus marinus* cells per sample. Three replicates for each cell number sample (10^2-10^6) were analyzed. The 95% confidence intervals are shown. The linear equation and the R^2 value are given for the regression.



$f(x) = 1.215\,x + 0.273 \qquad R^2 = 0.942$

Figure 13. Hemolymph standard curve depicts the relationship between the log of the amount of DNA (fg) determined by QCPCR and the log of the number of *Perkinsus marinus* cells per sample. Three replicates for each cell number sample (10^2-10^6) were analyzed. The 95% confidence intervals are shown. The linear equation and the R^2 value are given for the regression.



$$f(x) = 0.903\ x + 3.038 \qquad R^2 = 0.823$$

Table 1 lists the *P. marinus* infection diagnosis results for all 5 procedures which were performed on each oyster. For the QCPCR results, any number greater than 0 but less than 1 was assigned a value of 1 cell. Ray's FTM assay diagnosed only 19 infections while the body burden whole oyster tissue assay diagnosed infections in 24 of the 25 oysters. The FTM hemolymph assay detected 22 infections. QCPCR with both the gill and mantle tissue sample and the hemolymph sample detected 24 infections. Six oysters were diagnosed as uninfected by Ray's FTM assay. Four of these six oysters (Oysters 22, 43, 44, and 49) were diagnosed as positive by all of the other methods employed. Another oyster (Oyster 46) diagnosed as negative by RFTM was also found to be negative by the FTM hemolymph assay but positive by all other methods. Finally, Oyster 48 was found to be uninfected by Ray's FTM assay and QCPCR gill and mantle assay although all three other methods resulted in positive diagnoses.

Oyster 3 was found to be infected by all methods employed except for the FTM hemolymph assay. Oyster 36 was positive for *P. marinus* infection by Ray's FTM assay and QCPCR gill and mantle assay but was found to be negative by FTM hemolymph, body burden, and QCPCR hemolymph. All of the other 17 oysters were found to be infected by all five methods employed.

Regression analysis was performed on the results from diagnosis methods in pairs (Figure 14). The data from the body burden, FTM hemolymph, QCPCR gill and mantle, and QCPCR hemolymph assays were log transformed prior to analysis. Values that appeared in Table 1 as positive by QCPCR but below the level of quantitation were not included in the regression analysis. Table 2 lists the R^2 values obtained from the regression and the p values obtained from the ANOVA. All pairs analyzed resulted in highly significant correlations ($p<0.0001$). The relatively high R^2 values (range 0.568 to 0.875) indicated a good fit of the data with the regression curve and that much of the variance of the dependent variable could be accounted for by the independent variable.

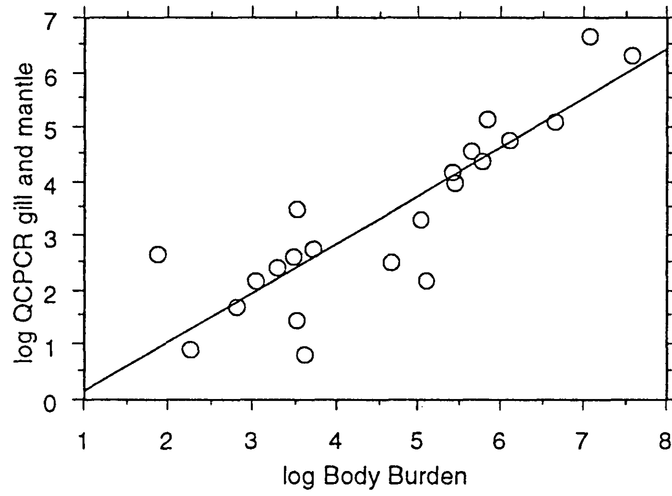
Table 1. *Perkinsus marinus* infection diagnosis by Ray's FTM assay, body burden assay (#cells/g), QCPCR with gill and mantle tissue (#cells/g), FTM hemolymph assay (#cells/ml), and QCPCR with hemolymph (#cells/ml).

Oyster #	RFTM rank	Body Burden #cells/g	QCPCR gill and mantle #cells/g	FTM hemolymph #cells/ml	QCPCR hemolymph #cells/ml
1	1	3332	3090	27	2
2	5	11379338	4466840	3689	920
3	0.5	76	468	0	1
4	0.5	3397	30	13	1
5	0.5	2040	275	7	1
6	4	595104	22909	4622	224
12	1	102237	2042	7	22
14	1	5437	589	30	1
16	5	1235772	60256	62833	1125
17	1	427083	37154	7200	398
18	5	4217826	128825	17778	2618
19	3	663727	134896	22000	867
21	5	38725490	2187760	5866667	20512
22	0	44538	324	40	1
23	2	274296	9333	7222	58
26	4	258763	15136	84444	12503
31	1	3219	427	37	25
34	0.5	120622	158	27	15
36	1	0	+ [a]	0	0
38	0.5	185	8	7	1
43	0	640	49	7	1
44	0	1087	155	13	1
46	0	4167	7	0	1
48	0	18	0	3	1
49	0	14	+ [a]	40	+ [a]

[a] denotes a positive oyster sample by QCPCR; however, the sample was below the level of quantitation (<0.005 fg)

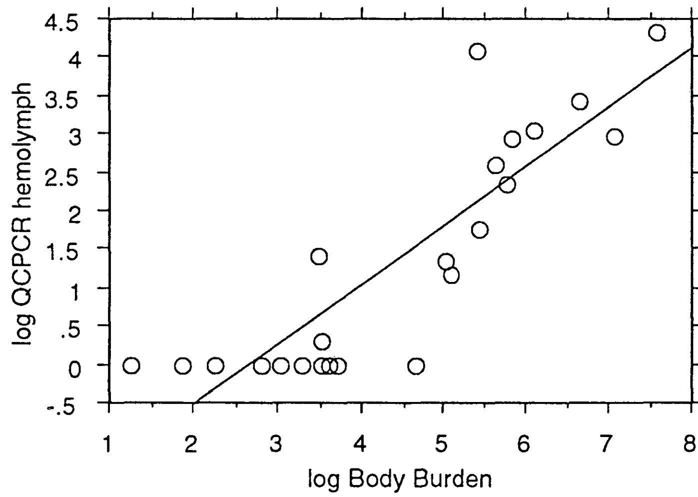
Figure 14. Regression analysis for pairwise comparison of *Perkinsus marinus* diagnostic methods: Ray's FTM assay, whole oyster body burden assay, FTM hemolymph assay, QCPCR gill and mantle assay, and QCPCR hemolymph assay. Regressions were performed on the number of *P. marinus* cells calculated using each of the methods. Regression equations and R^2 values are given for each of the graphs. Graphs depict log transformed data from all assays except the Ray's FTM rank data. (A) QCPCR gill and mantle vs. body burden. (B) QCPCR hemolymph vs. body burden. (C) QCPCR gill and mantle vs. Ray's FTM rank. (D) QCPCR hemolymph vs. Ray's FTM rank. (E) QCPCR gill and mantle vs. FTM hemolymph. (F) QCPCR hemolymph vs. FTM hemolymph. (G) QCPCR gill and mantle vs. QCPCR hemolymph.

A



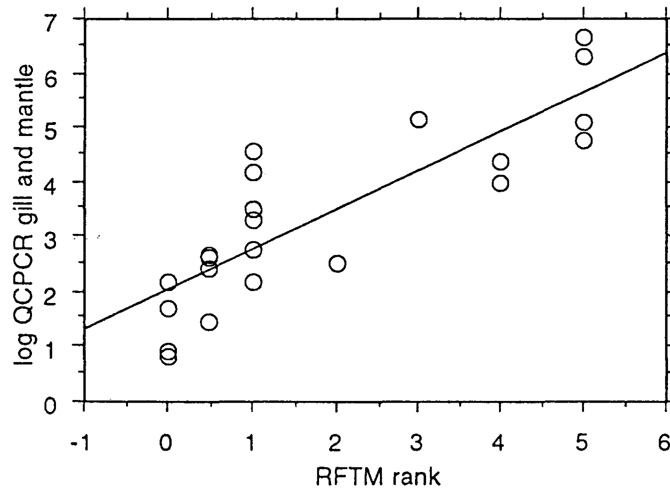
$f(x) = 0.899\ x - 0.769 \qquad R^2 = 0.758$

B



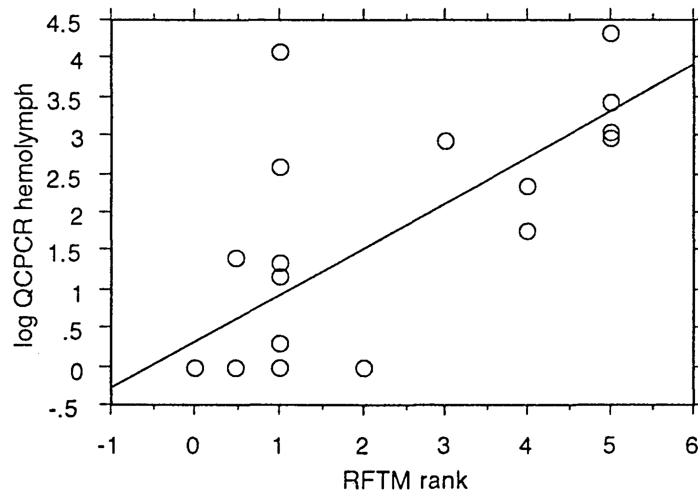
$f(x) = 0.778\ x - 2.092 \qquad R^2 = 0.753$

C



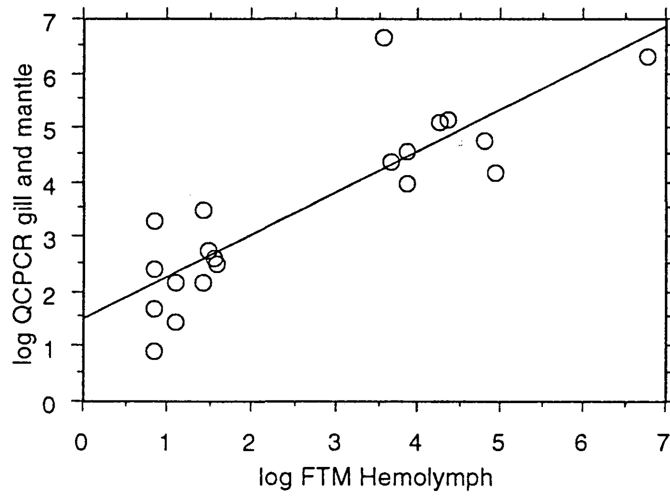
$f(x) = 0.727\,x + 2.019$ $R^2 = 0.716$

D



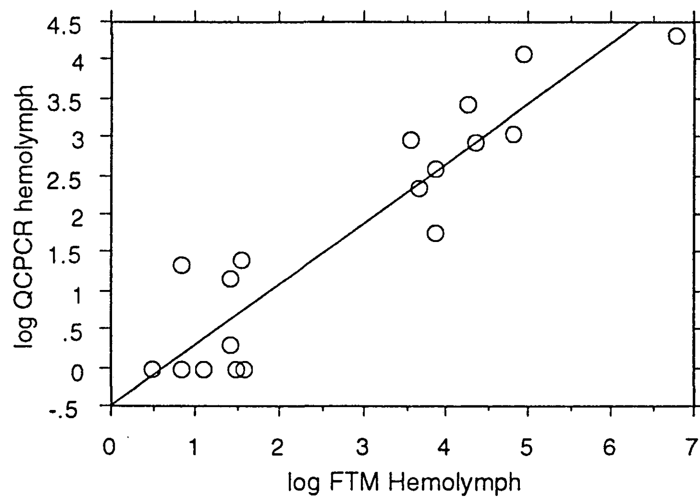
$f(x) = 0.604\,x + 0.302$ $R^2 = 0.568$

E



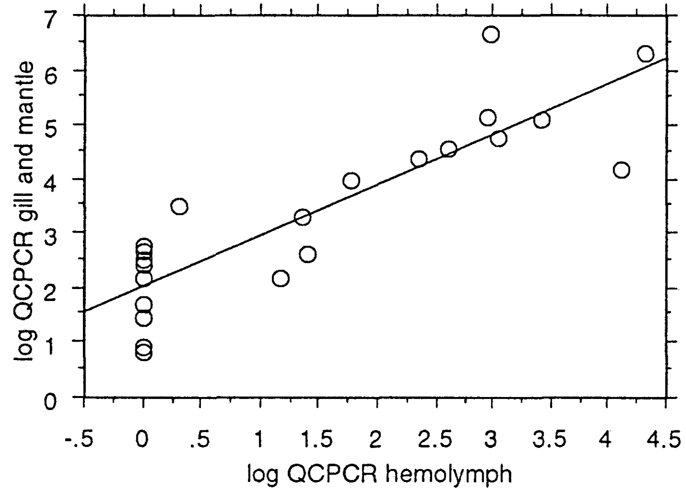
$f(x) = 0.761\ x + 1.511$ $R^2 = 0.735$

F



$f(x) = 0.786\ x - 0.497$ $R^2 = 0.875$

G



$f(x) = 0.928\ x + 2.035$ $R^2 = 0.752$

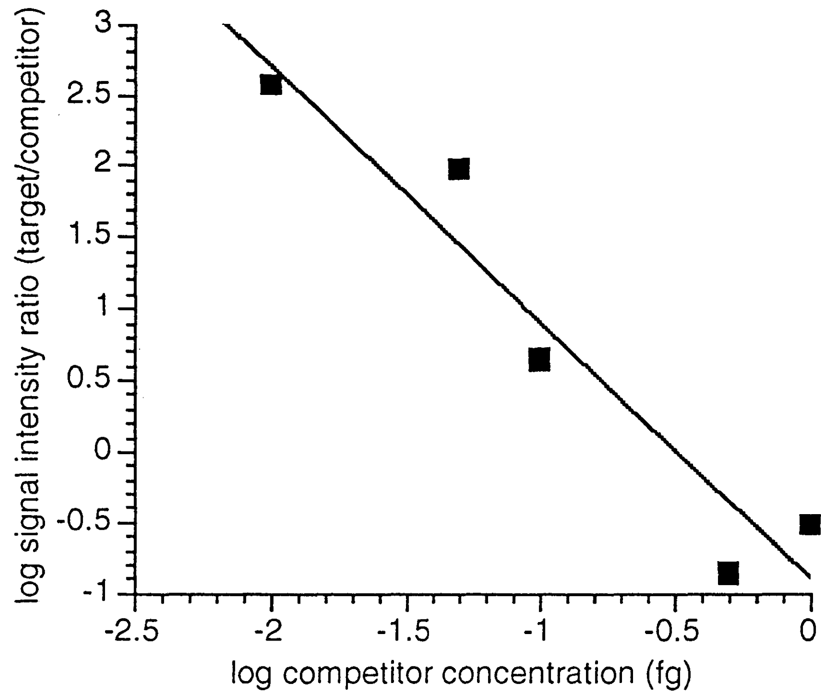
Table 2. Comparison by regression of all *Perkinsus marinus* diagnostic assays. All data were log transformed before regression analysis except for RFTM rank data. R^2 values and p values are given for each comparison.

	RFTM rank	log body burden	log QCPCR gill and mantle	log FTM hemolymph	log QCPCR hemolymph
RFTM rank	----	----	----	----	----
log body burden	R^2=0.713 p<0.0001	----	----	----	----
log QCPCR gill and mantle	R^2=0.716 p<0.0001	R^2=0.758 p<0.0001	----	----	----
log FTM hemolymph	R^2=0.637 p<0.0001	R^2=0.675 p<0.0001	R^2=0.735 p<0.0001	----	----
log QCPCR hemolymph	R^2=0.568 p<0.0001	R^2=0.753 p<0.0001	R^2=0.752 p<0.0001	R^2=0.875 p<0.0001	----

York River Water Samples

Figure 15 illustrates the raw QCPCR data for the September 26, 1997 water sample. The equivalence point was found to be at 0.892 fg DNA. After correcting for dilutions (2000), sample size (20), and genomic correction for the enzyme (7.296), the sample was found to have 260466.271 fg (or 260.5 pg) *P. marinus* DNA per liter of water. For the October 13, 1997 sampling date, triplicate one liter water samples were analyzed by QCPCR. Two of the three samples were diagnosed as positive for *P. marinus* presence; however, these samples were below the limit of quantitation (< 0.005 fg DNA). No *P. marinus* cells were found in an additional three replicate water samples taken on October 13, 1997 and processed by the modified RFTM body burden protocol.

Figure 15. QCPCR with genomic target DNA isolated from York River sample (#2) from September 26, 1997. Plasmid competitor DNA concentrations were 1 fg/µl, 0.5 fg/µl, 0.1 fg/µl, 0.05 fg/µl, 0.01 fg/µl and 0.005 fg/µl. The linear equation and the R^2 value are given for the regression.



f(x) -1.79744 x - 0.887813 $R^2 = 0.914$

DISCUSSION

Quantitative competitive PCR was employed to detect and quantitate *Perkinsus marinus* cells in oyster tissues and in environmental water samples. Primers amplified a 1210 bp region of the small subunit rRNA gene and the first internal transcribed spacer region of *P. marinus*. A competitor molecule was constructed which was homologous to the target sequences except for a 13 bp deletion. A two phase system allowed accurate quantitation of *P. marinus* DNA in 25 oyster samples within the linear range. The advantage of QCPCR over noncompetitive quantitative PCR stems from the competitor and the target being amplified in the same reaction tube and thus being equally affected by variations in inhibitors, temperature cycling, and reaction component concentrations.

In order to quantitate DNA based on the known concentration of the competitor, the amplification efficiency for both the target and the competitor must be assessed under the assay conditions. According to Cross (1995), the slope of the plot of the log of the signal intensity ratio (target/competitor) versus the log of the competitor concentration should be equal to -1 when the amplification efficiencies are equivalent; however, slight deviations from this value will not affect the determination of the equivalence point as long as a series of reactions which span the equivalence point is performed. A parallel shift of the curve can result from a difference in amplification efficiency in all of the reactions in the series (Raeymakers, 1995). Slight deviations from a slope of -1 in the graphs presented here may be accounted for by several sources of error; however, quantitation would still be accurate since a series of reactions which spanned the equivalence point was performed. Pipeting appeared to be a major source of error in the second phase series of

QCPCR reactions. Small pipeting errors may have altered the QCPCR series of reactions due to the fact that the competitor dilution series concentrations were in 2-fold or 5-fold increments. Replicate QCPCR reactions were performed on samples when error seemed extreme as judged by banding patterns which were inconsistent across the series of reactions. In these cases, data from all replicates were used in the determination of the equivalence point. Genomic target DNA as well as the plasmid competitor DNA were digested with a restriction endonuclease in order to make the sequences more accessible to the DNA polymerase in the PCR. This digestion procedure was shown to make the amplification efficiencies of genomic and plasmid DNA more equivalent. Another source of error may have been the degradation of the competitor molecule over time. While the concentration of the competitor was monitored over time, quantitation by the fluorometer may not have been sensitive enough to detect minute changes which may have affected the QCPCR. Overall, it can be concluded that the competitor plasmid and the target DNA amplified with equivalent amplification efficiencies after digestion with the restriction endonuclease *Xba*I under the conditions described for this assay.

The generation of a standard curve which relates the number of *P. marinus* cells to the amount of DNA present in the sample is essential for interpreting the QCPCR results. This standard curve prevents error associated with loss of DNA due to extraction and purification. While this loss is impossible to control, the use of the same isolation protocols for both the standard and the sample limits the effect of this loss. One potential problem for quantitation of *P. marinus* stems from the use of cultured cells in the standard curve. Although a genomic conversion factor was used to correct for differences in the number of target sequences between equal amounts of plasmid and genomic target DNA, the copy number of the rRNA gene in cultured cells versus natural cells is unknown. Estimations of copy number are difficult since the genome size of *P. marinus* has not been reported. QCPCR results must be interpreted using a standard

curve to account for these discrepancies although wildtype cells may, in fact, be different from the cultured cells. It has been suggested by Reece et al. (1997a) that *P. marinus* cultured cells are diploid; however, these cultured cells may be altered from their original state by propagation under culture conditions. Alterations in the ploidy of cultured cells from their natural state would alter the standard curve and in turn skew the *P. marinus* infection results. Furthermore, it must be assumed that the number of copies of the rRNA gene within the genome remains the same throughout the life of the organism. This assumption is difficult to prove at this time and differences may again skew the PCR results.

The use of the QCPCR assay allowed quantitation of *P. marinus* with extreme sensitivity and specificity. In a previous semi-quantitative PCR assay, Marsh et al. (1995) estimated concentrations of DNA by end-point dilution. While this method did offer the first documented molecular quantitation for *P. marinus*, the assay did not offer absolute quantitation as does the QCPCR. Furthermore, the limit of detection by the semi-quantitative PCR assay was 100 fg with the Southern blot and 10 fg with the dot blot. The limit of detection with the QCPCR assay was 0.005 fg *P. marinus* DNA in 0.4 µg of oyster tissue DNA. Thus, the QCPCR assay was found to be more sensitive. Another disadvantage of the semi-quantitative assay was the use of the ^{32}P radioisotope for visualization. The use of the LiCor automated sequencer to detect fluorescent dye-labeled primer eliminates the need for the dangerous isotope to be used. The automated sequencer detection of fluorescently labeled primers has been shown to be more sensitive than the ethidium bromide staining of agarose gels (Jenkins, 1994). Typically, detection of a product band on an agarose gel by ethidium bromide staining requires the presence of 2-5 ng of the PCR product. The standard curve results showed that the use of 1/50 of a DNA preparation of 100 cultured *P. marinus* cells was clearly within the range of quantitation. Thus, the automated sequencer allowed detection of PCR product amplified

from DNA from ≤ 2 cells in a background of 5 mg oyster tissue. This sensitivity level is extremely important in diagnosing rare or early infections in oyster tissues or when tissue samples are limited such as in juveniles. Furthermore, this level of sensitivity minimizes the chance for false negative diagnoses which are common to Ray's FTM assay and the FTM hemolymph assay (Bushek et al., 1994).

The sensitivity of the assay was maximized by using the Expand High Fidelity system; however the cost of this enzyme precluded its use for all reactions involved in the development of the assay. Therefore, Expand High Fidelity was used in the second phase QCPCRs only. Different genomic correction factors for the two enzymes suggest that amplification does not proceed with the same reaction kinetics in the presence of the different DNA polymerases. The smaller correction factor value found for the Expand High Fidelity PCR system may be explained by the increased performance of the enzymes. Previous investigations by Schwieger and Tebbe (1997) have demonstrated that the higher PCR product yields were obtained from low copy number DNA templates with the use of the Expand High Fidelity PCR system than with just *Taq* polymerase. False negatives are therefore reduced. Furthermore, this PCR system allows the amplification of template DNA which might be difficult to amplify using *Taq* polymerase alone. Results, however, cannot be compared between the estimation obtained using the first phase reactions with Ampli*Taq* and the value at the equivalence point from the second phase reactions with Expand High Fidelity.

Specificity of the primers at the species level is another major attribute of this assay. The primers did not amplify DNA isolated from the other *Perkinsus* species tested while they did amplify DNA from all geographic isolates of *P. marinus* tested. It has been suggested that these geographic isolates represent the genetic variation among available *P. marinus* isolate cultures (Reece et al., 1997). Specificity at the species level is unique to this method whereas Ray's FTM method (Ray, 1952) and the antibody flow cytometry

method (Dungan and Roberson, 1993; Ragone Calvo et al., 1995) have not demonstrated specificity to the species level. Species level specificity eliminates false positive results. It was shown that dinoflagellates and *Perkinsus atlanticus* did not amplify with these primers. Thus, unless sample to sample contamination occurred, it is very unlikely that a PCR product would be detected in the absence of *P. marinus* cells. Furthermore, employing specific molecular diagnostics allows for the detection of the parasite in all of its life stages.

Another advantage of QCPCR for the quantitation of *Perkinsus marinus* is objectivity. The accepted method of infection diagnosis in oyster tissue is Ray's FTM method which relies on estimates of *P. marinus* cells per field and then an assignment of an infection intensity. Using QCPCR, the number of cells from a known amount of tissue is determined mathematically. Additional problems with all FTM methods are that enlarged parasites do not consistently stain with Lugol's iodine and that parasite cells do not enlarge uniformly (Bushek et al., 1994). As a result, the investigator must make judgments when assigning infection intensities or counting cells.

The whole oyster body burden assay also gives results in objective wet standardized cell counts. Previously, the body burden assay was shown to be the most sensitive and most accurate diagnostic method available (Bushek et al., 1994). This is to be expected since the entire tissue is used for the procedure and all parasites which enlarge in the media and take up stain are subsequently counted. Therefore, the body burden assay may currently be considered the "gold standard" *P. marinus* diagnostic assay. Both the body burden and the QCPCR gill and mantle assay detected infections in 24 of 25 oysters. DNA for the QCPCR assay was isolated from only 0.25 grams of gill and mantle tissue. In patchy or localized infections, the chance of sampling 0.25 grams of uninfected tissue seems high although no evidence to support this hypothesis was seen in this study. In addition, even heavy infections may not be uniformly distributed throughout the oyster

tissues. Sampling may have been the primary cause for discrepancies between the body burden parasite counts and the estimates of the number of parasites calculated from the gill and mantle QCPCR assay. The relatively small amount of tissue required for the sensitive QCPCR assay, however, allows for other investigative procedures to be employed with the same oyster. This advantage becomes very valuable in laboratory settings where disease research involves monitoring infection levels and disease progression in addition to biochemical, histological, or other investigative procedures.

The presence of *P. marinus* cells in the gill, mantle, and digestive tissues depends on route of infection and disease progression stage (Fisher and Oliver, 1996). It has been demonstrated by Bushek et al. (1994) that combining rectal and mantle tissue for diagnosis using FTM methods decreased the chances for false negative diagnoses. Provided that *P. marinus* infections become established in the gut, the rectum is a sensitive tissue for disease monitoring. The body burden assay sampled the gill, mantle, rectum, and visceral mass tissues. This sampling of the gut, rectum, and visceral mass may have increased the sensitivity of the body burden relative to the QCPCR assay which only sampled gill and mantle tissue. Although the inclusion of rectal tissue may increase the sensitivity of this QCPCR assay for extremely low level infections in other oyster samples, it was not used for these samples due to the presence of PCR inhibitors in the rectal tissue DNA preparations.

QCPCR results were given as numbers of *P. marinus* cells per gram of tissue or per milliliter of hemolymph. These values were calculated based on the standard curve linear regression equation. The 95% confidence intervals which were calculated for these standard curves illustrate the variance around the slope of the line. The outer limits represent the possible range of cells that may have been calculated for the oyster tissue samples based on the QCPCR results and the standard curve. Thus, the QCPCR results

are a function of the regression equation and this fact may account for some of the difference seen between the QCPCR assays and the traditional FTM assays.

Furthermore, Ray's FTM assay categorizes infections based on parasite density (Bushek et al., 1994). Therefore, the variation seen in the regression plots comparing the QCPCR methods with the Ray's FTM assay infection intensity ranks (Figures 14C and 14D) may stem directly from the wide range of parasite densities which correspond to each of the ranks. Since infections are often patchy especially in the gill and mantle tissue, sampling different locations in the oyster may have encouraged discrepancies in the parasite burdens reported by each of these methods.

Overall, the QCPCR hemolymph assay appeared to be much more sensitive than the hemolymph FTM assay. The hemolymph FTM assay has typically been more accurate in detecting low level infections in oysters than Ray's FTM method (Gauthier and Fisher, 1990) although the hemolymph FTM assay is not as accurate an estimate of infection intensity as the body burden assay. The FTM hemolymph assay detected infections in 22 of 25 oysters whereas the QCPCR hemolymph assay detected infections in 24 of 25 oyster samples. Only one oyster was diagnosed as negative by the QCPCR hemolymph assay and it was also negative in the body burden assay. Since the body burden assay has proved most effective in diagnosing infections, a highly significant correlation ($p<0.0001$) between the QCPCR hemolymph assay and the body burden assay combined with a R^2 value of 0.753 lends support to the applicability of this QCPCR hemolymph assay for disease monitoring. The primary benefit to using a hemolymph sample is the non-destructive nature of the sampling. The hemolymph assay reflects systemic infections as opposed to localized tissue infections and thus may be important for monitoring infection establishment and not just presence of *P. marinus* in the oyster. Also, oysters can be bled several times over the course of an infection to monitor disease progression.

The QCPCR assay was used to quantitate *P. marinus* cells in environmental water samples from the York River. Quantitation was possible for one water sample, and positive but non-quantifiable results were obtained for 2 of the other 3 samples. Inhibitors present in the water column cause extreme problems for PCR. Water samples commonly contain humic substances, polysaccharides, and various other macromolecules that prevent efficient amplification (Leser, 1995). Often these inhibitory molecules can be removed from the DNA via CTAB extractions so that amplification can occur; however, even after this purification step, the detection limit for competitive PCR in water was found to be lower than expected. Leser et al (1995) found that the detection limit was lower even than that for sediment samples.

The variability between samples collected 17 days apart (September 26, 1997 and October 13, 1997) may reflect a difference in *P. marinus* abundance in the water column. *P. marinus* has been found in the water column at abundances of 50 to 590 cells per liter of water in September and October (Ragone Calvo et al., 1995). If these estimated abundances hold true on average, the number of *P. marinus* cells in the water column at this time of year is certainly within the detection limits of this QCPCR assay. Based on the fact that infected oysters were held in trays adjacent to the sampling site, the presence of some *P. marinus* cells in the water column seems feasible. A more likely explanation for the discrepancy in results between sampling dates is the presence of inhibitory molecules in the water sample. Humic substances may have been resuspended in the water column on the October date due to storm events or even activity on the river adjacent to the pier. It was noted that even amplification of the competitor was inhibited by the presence of undiluted York River water DNA on this sample date and that diluting the York River water DNA sample relieved this inhibition to some degree. This necessary dilution to allow amplification has been reported for sediment extract DNA samples when humic substances were present (Tsai and Olson, 1992). As a result, a

standard curve for interpretation of number of cells present per liter of water must be prepared with care since inhibitory molecules will affect the overall amplification and may change with seasonal conditions.

Using the QCPCR assay to monitor the *Perkinsus marinus* abundance in water samples is important since infective stages of the parasite are waterborne. A means for monitoring *P. marinus* specifically in the water column has not been documented previously. Although Ragone Calvo et al. (1995) labeled *P. marinus* cells from water column samples with antibodies, the specificity of the polyclonal antibodies has not been verified. At best, these antibodies detect hypnospores of three *Perkinsus* spp. (*P. marinus*, *P. atlanticus*, and *P. olseni*). Furthermore, the common RFTM methods are not easily applied to water samples and in fact *P. marinus* cells from the water column may not enlarge in RFTM. Attempts to utilize this method to monitor the water column were not successful.

For future use of this assay, it is recommended that quantitation be done over a broader linear range. For example, using competitor concentrations ranging from 50 fg to 0.005 fg might provide sufficient information to monitor infection levels. At the same time, error due to pipeting and limited competitor degradation would be minimized. Possibly, this amended assay may eliminate the need for replicates of the same DNA prep to be quantitated which would save both time and money. As for the need to quantitate on a linear curve, the points where only competitor or only target amplify are not used in the analysis since product intensity ratios are plotted. Thus, the curve will not be sigmoidal in nature despite the broad range of competitor used.

In conclusion, quantitative competitive PCR allowed for the quantitation of *P. marinus* DNA in oyster tissues and water samples. The disadvantages of this assay are the expense of the polymerase and the equipment as well as the molecular biology expertise required to perform the assay. In addition, PCR inhibitors in oyster rectal tissue and in

York River water present a challenge to the preparation of amplifiable DNA. The advantages of this QCPCR assay include its exceptional sensitivity using only small tissue samples as well as its species level specificity. The QCPCR hemolymph assay provides a non-destructive, repeatable method for determining pathogen infection in the oyster. In addition, the potential for analyzing water samples for *P. marinus* cells is exciting in the face of necessary transmission dynamics studies.

LITERATURE CITED

Andrews, J. D. 1988. Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effects on the oyster industry. American Fisheries Special Publication 18:47-63.

Andrews, J. D. and W. G. Hewatt. 1957. Oyster mortality studies in Virginia. II. The fungus disease caused by *Dermocystidium marinum* in oysters of the Chesapeake Bay. Ecological Monographs 27:1-23.

Arakawa, C. K., R. E. Deering, K. H. Higman, K. H. Oshima, P. J. O'Hara, and J. R. Winton. 1990. Polymerase chain reaction (PCR) amplification of a nucleoprotein gene sequence of infectious hematopoietic necrosis virus. Diseases of Aquatic Organisms 8:165-170.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl. 1988. Preparation and analysis of DNA. In Current Protocols In Molecular Biology, F. M. Ausubel (ed.). John Wiley and Sons, Inc. New York, NY. pp. 2.4.1- 2.4.5.

Barlough, J. E., T. S. McDowell, A. Milani, L. Bigornia, S. B. Slemenda, N. J. Pieniazek, and R. P. Hedrick. 1995. Nested polymerase chain reaction for the detection of *Enterocytozoon salmonis* genomic DNA in chinook salmon *Onchorhynchus tshawytscha*. Diseases of Aquatic Organisms 23:17-23.

Brown, L. L., G. K. Iwama, T. P. T. Evelyn, W. S. Nelson, and R. P. Levine. 1994. Use of the polymerase chain reaction (PCR) to detect DNA from *Renibacterium salmoninarum* within individual salmonid eggs. Diseases of Aquatic Organisms 18:165-171.

Brown, L. L., T. P. T. Evelyn, G. K. Iwama, W. S. Nelson, and R. P. Levine. 1995. Bacterial species other than *Renibacterium salmoninarum* cross-react with antisera against *R. salmoninarum* but are negative for the p57 gene of R. salmoninarum as detected by the polymerase chain reaction (PCR). Diseases of Aquatic Organisms 21:227-231.

Burreson, E. M., R. S. Alvarez, V. V. Martinez, and L. A. Macedo. 1994. *Perkinsus marinus* (Apicomplexa) as a potential source of oyster *Crassostrea virginica* mortality in coastal lagoons of Tabasco, Mexico. Diseases of Aquatic Organisms 20:77-82.

Burreson, E. M. and L. M. Ragone Calvo. 1996. Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay with emphasis on data since 1985. Journal of Shellfish Research 15:17-34.

Bushek, D. 1994. Dermo disease in American oysters: genetics of host-parasite interactions. Ph.D. dissertation, Rutgers-The State University, New Brunswick, N.J.

Bushek, D., S. E. Ford, and S. K. Allen. 1994. Evaluation of methods using Ray's fluid thioglycollate medium for diagnosis of *Perkinsus marinus* infection in the eastern oyster, *Crassostrea virginica*. Annual Review of Fish Diseases 4:201-217.

Choi, K. S., E. A. Wilson, D. H. Lewis, E. N. Powell, and S. M. Ray. 1989. The energetic cost of *Perkinsus marinus parasitism in oysters:* quantification of the thioglycollate method. Journal of Shellfish Research 8:125-131.

Chu, F. E. 1996. Laboratory investigations of susceptibility, infectivity, and transmission of *Perkinsus marinus* in oysters. Journal of Shellfish Research 15:57-66.

Clementi, M., S. Menzo, P. Bagnarelli, A. Manzin, A. Valenza, and P. E. Varaldo. 1993. Quantitative PCR and RT-PCR in virology. PCR Methods and Applications 2:191-196.

Cottrez, F., C. Auriault, A. Capron, and H. Groux. 1994. Quantitative PCR: validation of the use of a multispecific internal control. Nucleic Acids Research 22:2712-2713.

Craig, A., E. N. Powell, R. R. Fay, and J. M. Brooks. 1989. Distribution of *Perkinsus marinus* in Gulf Coast oyster populations. Estuaries 12:82-91.

Cross, N. C. P. 1995. Quantitative PCR techniques and applications. British Journal of Haematology 89:693-697.

Darnell, J., H. Lodish, and D. Baltimore. 1990. Molecular Cell Biology. 2nd Edition. New York, NY. Scientific American Books, Inc.

Dungan, C. F. and B. S. Roberson. 1993. Binding specificities of mono- and polyclonal antibodies to the protozoan oyster pathogen, *Perkinsus marinus*. Diseases of Aquatic Organisms 15:9-22.

Ehrlich, H. A., D. Gelfand, and J. J. Sninsky. 1991. Recent advances in the polymerase chain reaction. Science 252:1643-1651.

Ferre, F. 1992. Quantitative or semi-quantitative PCR: reality versus myth. PCR Methods and Applications 2:1-9.

Fisher, W. S. and L. M. Oliver. 1996. A whole-oyster procedure for diagnosis of *Perkinsus marinus* disease using Ray's fluid thioglycollate culture medium. Journal of Shellfish Research 15:109-117.

Flores, B. S., M. E. Siddall, and E. M. Burreson. 1996. Phylogeny of the Haplosporidia (Eukaryota:Alveolata) based on small subunit ribosomal RNA gene sequence. Journal of Parasitology 82:616-623.

Fong, D., R. Rodrigues, K. Koo, J. Sun, M. L. Sogin, D. Bushek, D. T. J. Littlewood, and S. E. Ford. 1993. Small subunit ribosomal RNA gene sequence of the oyster parasite *Perkinsus marinus*. Molecular Marine Biology and Biotechnology 2:346-350.

Ford, S. E. 1996. Range extension by the oyster parasite *Perkinsus marinus* into the northeastern United States: response to climate change? Journal of Shellfish Research 15:45-56.

Gauthier, J. D. and W. S. Fisher. 1990. Hemolymph assay for diagnosis of *Perkinsus marinus* in oysters *Crassostrea virginica*. Journal of Shellfish Research 9:367-372.

Gauthier, J. D. and G. R. Vasta. 1993. Continuous in vitro culture of the eastern oyster parasite *Perkinsus marinus*. Journal of Invertebrate Pathology 62:321-323.

Gilliland, G., S. Perrin, K. Blanchard, and H. F. Bunn. 1990a. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proceedings of the National Academy of Sciences, USA. 87:2725.

Gilliland, G., S. Perrin, and H. F. Bunn. 1990b. Competitive PCR for quantitation of mRNA. In PCR Protocols: A Guide to Methods and Applications, M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White(Eds.), Academic Press, Inc., San Diego, p. 60-69.

Goggin, C. L. 1994. Variation in the two internal transcribed spacers and 5.8S ribosomal RNA from five isolates of the marine parasite *Perkinsus* (Protista, Apicomplexa). Molecular and Biochemical Parasitology 65:179-182.

Goggin, C. L. and S. C. Barker. 1993. Phylogenetic position of the genus *Perkinsus* (Protista, Apicomplexa) based on small subunit ribosomal RNA. Molecular and Biochemical Parisitology 60:65-70.

Goodwin, D. C. and S. B. Lee. 1993. Microwave miniprep of total genomic DNA from fungi, plants, protists, and animals for PCR. BioTechniques 15:438-444.

Hahn, M., V. Dörsam, P. Friedhoff, A. Fritz, and A. Pingoud. 1995. Quantitative polymerase chain reaction with enzyme-linked immunosorbent assay detection of selectively digested amplified sample and control DNA. Analytical Biochemistry 229:236-248.

Hill, W. E., S. P. Keasler, M. W. Trucksess, P. Feng, C. A. Kaysner, and K. A. Lampel. 1991. Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. Applied and Environmental Microbiology 57:707-711.

Hudson, D. A. and R. D. Adlard. 1994. PCR techniques applied to *Hematodinium* spp. and *Hematodinium*-like dinoflagellates in decapod crustaceans. Diseases of Aquatic Organisms 20:203-206.

Innis, M. A. and D. H. Gelfand. 1990. Optimization of PCRs. In: PCR Protocols: A Guide to Methods and Applications, M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (eds.). Academic Press, Inc., San Diego, p. 3-12.

Jenkins, F. J. 1994. Basic methods for the detection of PCR products. PCR Methods and Applications 3:S77-S82

Kleinschuster, S. J., F. O. Perkins, M. J. Dykstra, and S. L. Swink. 1994. The *in vitro* life cycle of a *Perkinsus* species (Apicomplexa, Perkinsidae) isolated from *Macoma balthica* (Linneaus, 1758). Journal of Shellfish Research 13:461-465.

Ko, Y., S. E. Ford, and D. Fong. 1995. Characterization of the small subunit ribosomal RNA gene of the oyster parasite *Haplosporidium costale*. Molecular Marine Biology and Biotechnology 4:236-240.

Kreader, C. A. 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Applied and Environmental Microbiology 62:1102-1106.

LaPeyre, J. F., M. Faisal, and E. M. Burreson. 1993. In vitro propagation of the protozoan *Perkinsus marinus*, a pathogen of the eastern oyster, *Crassostrea virginica*. Journal of Eukaryotic Microbiology 40:304-310.

Leser, T. D. 1995. Quantitation of *Pseudomonas* sp. strain B13(FR1) in the marine environment by competitive polymerase chain reaction. Journal of Microbiological Methods 22:249-262.

Leser, T. D., M. Boye, and N. B. Hendriksen. 1995. Survival and activity of *Pseudomonas* sp. strain B13(FR1) in a marine microcosm determined by quantitative PCR and an rRNA-targeting probe and its effect on the indigenous bacterioplankton. Applied and Environmental Microbiology 61:1201-1207.

Levine, N. D. 1978. *Perkinsus* gen. n. and other new taxa in the protozoan Phylum Apicomplexa. Journal of Parasitology 64:549.

Lu, W., D. S. Han, J. Yuan and J. M. Andrieu. 1994. Multi-target PCR analysis by capillary electrophoresis and laser-induced fluorescence. Nature 368:269-271.

Mackin, J. G. 1956. *Dermocsytidium marinum* and salinity. Proceedings National Shellfisheries Association 46: 116-133.

Mackin, J. G. 1962. Oyster disease caused by *Dermocystidium marinum* and other microorganisms in Louisiana. Publication of the Institute of Marine Science, University of Texas 7:132-229.

Mackin, J. G., H. M. Owen, and A. Collier. 1950. Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassostrea virginica* (Gmelin). Science 111:328-329.

Mackin, J. G. and S. M. Ray. 1966. The taxonomic relationships of *Dermocystidium marinum* Mackin, Owen, and Collier. Journal of Invertebrate Pathology 8:544-545.

Marsh, A. G., J. D. Gauthier, and G. R. Vasta. 1995. A semiquantitative PCR assay for assessing *Perkinsus marinus* infections in the eastern oyster, *Crassostrea virginica*. Journal of Parasitology 81: 577-583.

Morrison, C. and F. Gannon. 1994. The impact of the PCR plateau phase on quantitative PCR. Biochimica et Biophysica Acta 1219:493-498.

Mullis, K. B. and F. A. Faloona. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods in Enzymology 155:335-350.

Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich. 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harbor Symposium of Quantitative Biology 51:263-273.

Nishizawa, T., K. Mori, T. Nakai, I. Furusawa, and K. Muroga. 1994. Polymerase chain reaction (PCR) amplification of RNA of striped jack nervous necrosis virus (SJNNV). Diseases of Aquatic Organisms 18:103-107.

Perkins, F. O. 1976. Zoospores of the oyster pathogen, *Dermocystidium marinum*. I. Fine structure of the conoid and sporozoan-like organelles. Journal of Parasitology 62:959-974.

Piatak, M. Jr., K. Luk, B. Williams, and J. D. Lifson. 1993. Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. BioTechniques 14:70-80.

Porcher, C., M.-C. Malinge, C. Picat, and B. Grandchamp. 1992. A simplified method for determination of specific DNA or RNA copy number using quantitative PCR and an automatic DNA sequencer. BioTechniques 13:106-113.

Raeymaekers, L. 1995. A commentary of the practical applications of competitive PCR. Genome Research 5:91-94.

Ragone, L. M. and E. M. Burreson. 1993. Effect of salinity on infection progression and pathogenicity of *Perkinsus marinus* in the eastern oyster, Crassostrea virginica (Gmelin). Journal of Shellfish Research 12:1-7.

Ragone Calvo, L. M. and E. M. Burreson. 1994. Characterization of overwintering infections of *Perkinsus marinus* (Apicomplexa) in Chesapeake Bay oysters. Journal of Shellfish Research 13:123-130.

Ragone Calvo, L. M., E. M. Burreson, C. F. Dungan, and B. S. Roberson. 1995. Field and laboratory study of the process and dynamics of *Perkinsus marinus* infection in the eastern oyster, *Crassostrea virginica*. Final Report No. NA47FL-0158. NOAA Oyster Disease Research Program. Virginia Institute of Marine Science, Gloucester Point, VA.

Ray, S. M. 1952. A culture technique for the diagnosis of infection with *Dermocystidium marinum* Mackin, Owen, and Collier in oysters. Science 114:360-361.

Ray, S. M. 1954. Biological studies of *Dermocystidium marinum*, a fungus parasite of oysters. Rice Institute Pamphlet, 114 pp.

Ray, S. M. and J. G. Mackin. 1954. Studies on transmission and pathogenicity of *Dermocystidium marinum*, I. Texas A&M Research Foundation Project 23 Technical Report. 1-11.

Reece, K. S., D. Bushek, and J. E. Graves. 1997a. Molecular markers for population genetic analysis of *Perkinsus marinus*. Molecular Marine Biology and Biotechnology 6:197-206.

Reece, K. S., M. E. Siddall, E. M. Burreson, and J. E. Graves. 1997b. Phylogenetic analysis of *Perkinsus* based on actin gene sequences. J. Parasitology 83:417-423.

Repp, R., A. Borkhardt, R. Gossen, J. Kreuder, J. Hammerman, and F. Lampert. 1995. Construction of RNA standards for high-resolution automatic product analysis in quantitative competitive RT-PCR. BioTechniques 19:84-90.

Saiki, R. K. 1990. Amplification of Genomic DNA. In PCR Protocols: A Guide to Methods and Applications, M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (eds.), Academic Press, Inc, San Diego, p. 13-20.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A laboratory manual. 2nd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences USA 74: 5463-5467.

Schwieger, F. and C. C. Tebbe. 1997. Efficient and accurate PCR amplification and detection of a recombinant gene in DNA directly extracted from soil using the Expand™ High Fidelity PCR System and T4 gene 32 protein. Biochemica 2:21-23.

Siddall, M. E., N. A. Stokes, and E. M. Burreson. 1995. Molecular phylogenetic evidence that the phylum Haplosporidia has alveolate ancestry. Molecular Biology and Evolution 12:573-581.

Siddall, M. E., K. S. Reece, J. E. Graves, and E. M. Burreson. 1997. 'Total evidence' refutes the inclusion of *Perkinsus* species in the phylum Apicomplexa. Parasitology 115:165-176.

Siebert, P. D. and J. W. Larrick. 1993. PCR MIMICS: Competitive DNA fragments for use as internal standards for quantitative PCR. BioTechniques 14:244-249.

Soong, N. W. and N. Arnheim. 1995. Quantitative PCR: Analysis of rare mitochondrial DNA mutations in central nervous system tissues. Methods in Neurosciences 26:105-128.

Stemmer, W. P. C. 1991. A 20-minute ethidium bromide/high-salt extraction protocol for plasmid DNA. BioTechniques 10:726.

Stokes, N. A., M. E. Siddall, and E. M. Burreson. 1995. Detection of *Haplosporidium nelsoni* (Haplosporidia: Haplosporidiidae) in oysters by PCR amplification. Diseases of Aquatic Organisms 23:145-152.

Sur, S., G. J. Gleich, M. E. Bolander, and G. Sarkar. 1995. Comparative evaluation of quantitative PCR methods. Methods in Neurosciences 26:147-160.

Thiery, R., P. Boutin, C. Arnauld, and A. Jestin. 1995. Efficient production of internal standard DNA for quantitative PCR using an automated sequencer. BioTechniques 18:212-213.

Tsai, T. L. and B. Olson. 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Applied and Environmental Microbiology 58:1249-1258.

Vivier, E. 1982. Réflexions et suggestions a propos del las systématique des sporozoaires: création d'une classe des *Hematozoa*. Protistologica XVIII:449-457.

Volety, A. K. and F. E. Chu. 1994. Comparison of infectivity and pathogenicity of meront (trophozoite) and prezoosporangiae stages of the oyster pathogen *Perkinsus marinus* in eastern oysters, *Crassostrea virginica* (Gmelin, 1791). Journal of Shellfish Research 13:521-527.

Wilson, K. 1994. Preparation of genomic DNA from bacteria. In: Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Stuhl (Eds.), Current Protocols in Molecular Biology. Volume 1. p. 2.4.1-2.4.5.

Zar, J. H. 1984. Biostatistical analysis. 2nd edition. Prentice-Hall, Inc. Englewood Cliffs, NJ.

Zimmermann, K. and J. W. Mannhalter. 1996. Technical aspects of quantitative competitive PCR. BioTechniques 21:268-279.

Zimmermann, K., D. Schögl, and J. W. Mannhalter. 1994. Hemi-nested quantitative competitive PCR of HIV-1. BioTechniques 17:440-442.

VITA

Heather A. Yarnall

Born in Mt. Lebanon, Pennsylvania, 28 February 1971. Graduated from Hickory High School, Hickory, North Carolina, in June 1989. Earned a B.S. in Biology with a minor in Chemistry from the University of North Carolina at Chapel Hill in May 1993. Employed as a Research Technician II in the Biology Department of the University of North Carolina at Chapel Hill from May 1993 to June 1994. Entered the Master of Science program at the College of William and Mary, School of Marine Science in August 1994. Employed as a graduate research assistant at the College of William and Mary, School of Marine Science from August 1994 to January 1998.