Validating a Quantitative Real-Time PCR Method to Detect Dermo (*Perkinsus marinus*) in Texas Oysters

A cooperative study between
Texas Parks & Wildlife Department and Texas Water Development Board

TWDB Contract #: 1004831018
Contract Time Period: Nov. 2009 through Jan. 2011

By

Texas Parks & Wildlife Department
Coastal Fisheries Division
Science and Policy Branch
Ecosystem Resources Program
Perry R. Bass Marine Fisheries Research Station

TPWD Project Contacts: Rebecca Hensley, Lance Robinson, Mark Fisher
TPWD Principal Investigators: Jan Culbertson, Joel Anderson, and William Karel
TAMUG Principal Investigator: Sammy Ray

And

Texas Water Development Board
Surface Water Resources Division
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TWDB Contract Manager: Carla Guthrie
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Final Report

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Executive Summary

The relationship between estuarine salinity and the persistence of the oyster-specific pathogen *Perkinsus marinus* has previously been noted by numerous authors (Hofstetter et al. 1965, Hofstetter 1977, Ray 1987, Soniat et al. 2005, La Peyre et al. 2003, 2009). This protozoan parasite thrives at elevated salinities and temperatures and is a major cause of eastern oyster (*Crassostrea virginica*) mortality (Ray 1954, Quick and Mackin 1971, La Peyre et al. 2003, 2009). Historically, *P. marinus* infections referred to as "Dermo disease" in Texas oyster populations have been monitored using Ray's fluid thioglycolate medium methodology (RFTM; Ray 1966). This traditional methodology requires tissue culture and staining of the *P. marinus* cells before categorically assigning a modified Mackin's scale disease code, based on the number of cells present in each assay (Ray 1966). The more intense the infection detected by RFTM assay the higher the Mackin scale disease codes assigned to each sample. Although RFTM has been widely utilized across the United States for monitoring *P. marinus* over the past 60 years, there are molecular methodologies such as real-time quantitative polymerase chain reaction (QPCR) assays which are able to determine the number of *P. marinus* cells present in each sample (Burreson 2008). QPCR quantitatively measures the threshold chemical reaction cycle time (Ct) for amplifying DNA of the target cells, rather than separating the data into discrete categories (Mackin scale disease codes). Thus QPCR provides continuous data sets making statistical manipulations more manageable (Choi et al. 1989). QPCR is also more sensitive to detecting low levels of Dermo infection than RFTM, which tends to underestimate the intensity of infection and may result in false negatives (Gauthier et al. 2006, Soniat 1996).

Texas Water Development Board (TWDB), with research and planning funds, supported a comparison study of two methodologies (QPCR and RFTM) in conjunction with Texas Parks and Wildlife Department (TPWD) Coastal Fisheries Division's ongoing Dermo monitoring program. The primary purpose of this study was to determine whether the two methodologies provide comparable results. The second objective was to standardize QPCR (Ct) results to the RFTM (Mackin scale of disease codes) results in order to preserve the continuity between historical and future data sets of Dermo infection in Texas oyster populations.

The study results confirmed QPCR is a viable alternative for monitoring and maintaining continuous records of Dermo infections in Texas oyster populations that is not dependent on the scientific investigator. Pearson's correlation coefficient analysis of all samples demonstrated there is a significant correlation ($P < 0.001$) between RFTM's categorical data (Mackin scale disease codes) and QPCR's continuous data (Ct). Linear regression of the average QPCR (Ct) values associated with each RFTM (Mackin scale disease code) category resulted in better model fit ($y = -4.891x + 38.58$, with $r^2 = 0.807$). Comparison of individual samples and individual bay systems indicate specific transport and storage protocols need to be maintained for standardization of tissue sample condition when comparing RFTM and QPCR results. Comparison of the two methodologies to detect Dermo infection in the same oyster demonstrates RFTM may be limited in detecting very small *P. marinus* cells during the winter months when temperatures are lower, QPCR was a more sensitive methodology for detecting low level amounts of Dermo infection under the same conditions. The study results demonstrates there are distinct geographic and hydrological differences between individual bay systems, individual reefs, and seasons that influence the amount of Dermo infection in each oyster population regardless of the method used to monitor it. Thus, sampling locations, storage protocols, tissue volume, geographic and hydrological influences are important factors to consider in any future Dermo infection monitoring program. In conclusion, the two methodologies provided comparable results, and QPCR is a suitable methodology for shifting techniques in order to preserve the continuity between historical and future data sets of Texas oyster populations.

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INRODUCTION

The future of freshwater inflows into Texas' estuaries is threatened by increased development of municipal, industrial and agricultural water needs. As such, biological metrics are needed that can accurately assess the impacts of reduced estuarine inflows and measure the impacts of elevated estuarine salinity at the organism level. One of these biological indicators being investigated for its long term relationship with freshwater inflows is the eastern oyster, *Crassostrea virginica.* Eastern oysters are considered one of the best ecological indicators of changes in estuaries because oyster are stationary reef-building organisms, and their populations only grow, reproduce and survive where environmental conditions are optimal (Chatry et al. 1983, Dame 1996, Gutierrez et al. 2003, Bergquist et al. 2006). Lengthy periods of drought and/or low freshwater inflows elevate estuarine salinities and result in increased oyster mortalities from predation and parasitism (Ray 1987). Alternatively, floods can reduce salinities long enough to cause massive oyster mortalities (Hofstetter 1977, Wilber 1992). However, high oyster mortalities associated with above average inflow conditions were often found to be synchronous with abundant oyster spat set since larvae prefer to set on "recent dead" shell substrate (Hofstetter 1977, Turner et al. 1994). Recent analysis of fisheries-independent data for Galveston Bay shows that oyster population abundance increases one- to two years after above average inflows and associated decreases in salinity (Buzan et al. 2009) implicating a complex relationship between oyster population viability and freshwater inflow events.

The eastern oyster is highly valued for both its ecological and economic role in estuaries along the northern Gulf of Mexico (Dame 1996, Bartol et al. 1999, Minello 1999, Plunket and La Peyre 2005, Coen and Grizzle 2007, Culbertson 2008, Buzan et al. 2009). In Texas, eastern oysters are the second most valuable commercial fishery. Recent commercial oyster landings have reported 5.8 million pounds of meat weight worth $19.1 million in 2010 (TPWD unpublished data 2010). Additionally, oysters and their reef formations are an essential component of the estuarine ecosystem and provide a variety of ecological functions. Oysters are dynamic engineers that secrete calcareous shells, and in so doing, create three dimensional structural habitats with interstitial heterogeneity necessary to maintain a diverse community of commensal, predatory and parasitic organisms that live, feed, or seek refuge on these reefs (Dame 1979, 1996, Bartol et al. 1999, Minello 1999, Zimmerman et al. 1989). Zimmerman et al. (1989) demonstrated that unique assemblages of juvenile macrofauna used oyster reefs more commonly than they did either salt marsh or unvegetated mud flat habitat. In terms of hydrologic effects, oysters exert "top-down" grazer control on increased phytoplankton biomass indirectly caused by anthropogenic effects, such as the increased levels of nitrogen and phosphorous available from nutrient-enriched runoff (Newell et al. 2005). Thus, oysters have both habitat-associated (indirect) effects as well as nutrient cycling (direct) effects on estuarine systems.

The relationship between estuarine salinity and the persistence of the oyster-specific pathogen *Perkinsus marinus* (referred to as "Dermo" disease) has previously been noted by numerous authors (Hofstetter et al. 1965, Hofstetter 1977, Ray 1987, Soniat et al. 2005, La Peyre et al. 2003, 2009). This protozoan parasite thrives at elevated salinities and temperatures and is a major cause of eastern oyster (*C. virginica*) mortality (Ray 1954, Quick and Mackin 1971, Gauthier et al. 1990, La Peyre et al. 2003, 2009). This parasite inhabits the immune cells of the oyster and suppresses the effectiveness of the oyster phagocytes; thereby overwhelming the oyster's immune system and rendering it unable to fight off other opportunistic marine organisms. The effects of *P. marinus* infection in *C. virginica* range from pale appearance of the digestive gland, reductions in condition index, impaired gametogenic development, reduced haemolymph protein concentrations and lysozyme activity, to severe emaciation, gaping, shrinkage of the mantle away from the outer edge of the shell, retarded growth, occasionally the presence of pus-like pockets, and death (Ford and Tripp 1996). The Eastern Oyster Biological Review Team (2007) reported this "parasite infects oysters in their first year of life and continues to proliferate causing up to 50% mortalities in oysters carrying the infection into their second summer season, 80-90% mortalities by the third year, with very few oysters that are infected with this disease organism surviving their fourth summer season." Dermo infection appears to increase and spread within an oyster population during higher salinity (>15 ppt) and higher temperature

(>25° C) combinations (Ray 1987, Song 1993, Soniat 1996, Kennedy et al. 1996). Dermo-related mortality generally peaks during the summer when environmental conditions are optimal for parasite growth, and these conditions can be exaggerated in the event of a drought (Burreson and Andrews 1988, Chu and La Peyre 1993, Chu et al. 1993). In contrast, colder temperatures generally reduce the spread and intensity of this pathogen as infection declines at temperatures below 15 to 20 °C (Ray 1987). Chu and Greene (1989) exposed prezoosporangia and zoospores from *P. marinus* to near freezing water temperatures and determined this parasite cannot withstand temperatures as low as 4 °C for more than four days and the zoospores died after one day when transferred from 28 to 4 °C.

P. marinus infections in oyster populations appear to diminish during repetitive and well-timed low salinity (freshet) events that prevent the spread of infection or at least maintain *P. marinus* at non-lethal intensities (La Peyre et al. 2003, 2009). Specifically, low salinity events (less than five ppt) decrease *P. marinus* infection intensities, even as temperatures exceed 20°C (La Peyre et al. 2009). Thus well-timed freshwater inflow events may provide a possible adaptive management approach to reducing *P. marinus* infection in oyster populations. However, large-scale climatic conditions, such as those associated with El Niño southern oscillation (ENSO) cycles, may also facilitate the initiation and progression of *P. marinus* infections along the coast of the Gulf of Mexico (Powell et al. 1992, 1996, Kim and Powell 1998, Soniat et al. 2005). Therefore the long-term variations in salinity combined with higher temperatures are a critical factor for predicting the prevalence and intensity of *P. marinus* in oyster populations along the Gulf coast. For this reason, fluctuations in the occurrence of *P. marinus* infection in oysters may be an important biological indicator that can be used to monitor estuarine health and the impacts of reduced freshwater inflows in Texas estuaries through time.

Historically, *P. marinus* infections in Texas oyster populations have been monitored in five bay system by Texas Parks and Wildlife Department (TPWD) Coastal Fisheries Division and Texas A&M University in Galveston (TAMUG) using Ray's fluid thioglycolate medium methodology (RFTM; Ray 1966). This traditional methodology requires tissue culture under anaerobic dark conditions and staining of the *P. marinus* cells before categorically assigning a modified Mackin's scale disease code to the number of cells present or density of cells in each assay (Ray 1966). More intense infections detected by RFTM assay results in a higher the Mackin scale disease codes assigned to each sample.

Although RFTM has been widely utilized across the United States for monitoring *P. marinus* over the past 60 years, this methodology cannot distinguish between multiple species of *Perkinsus*. Several molecular methodologies have recently been developed to assay *Perkinsus* intensity in both oyster samples (Yarnall et al. 2000, Gauthier et al. 2006) and in environmental water samples (Audemard et al. 2004). These molecular methodologies such as real-time quantitative polymerase chain reaction (QPCR) assays are able to determine the number of *P. marinus* cells present (Burreson 2008). QPCR methodology measures the threshold chemical reaction cycle time (Ct) for amplifying DNA of the target cells, rather than separating the data into discrete categories (Mackin scale disease codes). Thus QPCR methodology provides continuous data, making statistical manipulations more manageable (Choi et al. 1989). The QPCR methodology is also more sensitive to detecting low levels of Dermo infection than the RFTM methodology, which tends to underestimate the intensity of infection and may result in false negatives (Soniat 1996, Gauthier et al. 2006). RFTM and QPCR methodologies will be referred to as RFTM and QPCR unless otherwise noted in this document.

A major drawback for continuing to use RFTM is its dependence upon the expertise of the person quantifying the number of cells present in the mantle tissues and results can vary between individuals. To date all TPWD-TAMUG Dermo evaluations for Texas Bays have been quantified by Dr. Sammy Ray at TAMUG and any change to this protocol would introduce unknown variability into Dermo infection and could affect the comparability with historic data.

Parallel testing of both methodologies (RFTM and QPCR) was previously conducted by TPWD in a pilot study to quantify Dermo infection in oysters from Matagorda Bay in 2007 (TPWD unpublished data). This pilot study showed QPCR (Ct) values provided comparable results with RFTM (Mackin scale disease codes) readings. This pilot study also showed QPCR can reduce the time and laboratory effort necessary to assay Dermo disease intensity in individual oysters versus using traditional RFTM. Although the results of the pilot study showed potential benefits, additional evaluation of the QPCR used on samples from multiple reefs in multiple bay systems over a longer period of time would be required to consider this new methodology for implementation in the Coastal Fisheries Resource Management Program.

Purpose of Study

Texas Water Development Board (TWDB), with research and planning funds, supported a comparison study of QPCR and RFTM methodologies in conjunction with TPWD's Coastal Fisheries Division's ongoing Dermo monitoring program in five bay systems. The primary purposes of this contract study were:

1. Validate the two methodologies provide comparable results from oyster populations in five bay systems over a thirteen month study period.
2. Standardize QPCR (Ct) results to the RFTM (Mackin scale of disease codes) results in order to preserve the continuity between historical and future data sets of Dermo infection in Texas oyster populations.

In addition to the primary contract study purpose, TPWD had additional objectives for this study and continued to collect additional months of data after the TWDB contract study period ended in order to provide long term management recommendations. Those objectives include:

1. Identify potential modifications to the current Dermo monitoring sampling protocols (e.g., sample size, sample frequency, sample locations, and sample storage requirements).
2. Review efficacy of using the QPCR for continuing to monitor Dermo infection in Texas oyster populations in conjunction with ongoing water quantity assessments.
3. Assess long term trends of Dermo infection as a potential biological metric for correlating the ecological condition of Texas bays and estuaries under varying rates of freshwater inflow.

This study and the extended study provide an opportunity to assess the ecological and economic benefits of establishing a Dermo infection monitoring program in Texas oyster populations. The results of this assessment and any future management recommendations from this study could serve as the basis for implementation of a suite of sampling programs aimed at monitoring freshwater inflow effects on estuarine systems.

METHODS

Overview

TPWD has routinely monitored Texas oyster populations in multiple bay systems since 1986. These routine resource monitoring samples have also been evaluated for Dermo disease since 1998 using RFTM (Ray 1966). In the current study, TPWD compared the use of QPCR (Gauthier et al. 2006) side-by-side with traditional RFTM (Ray 1966) to assay Dermo disease in individual oysters collected from five separate bay systems. This portion of the study funded by TWDB was conducted over a thirteen month period between October 2009 and November 2010. TPWD continued to monitor Dermo disease for an additional ten months following the TWDB contract study period and those results are currently being reviewed. All data inclusive of the contract study results will be presented in a separate report.

Sample Locations

TPWD collected oysters from five separate bay systems (Sabine Lake, Galveston, Matagorda, Lavaca, and San Antonio Bays) for determining potential spatial differences in the results. Twelve primary target reef locations were selected based on their proximity or distance away from freshwater inflow sources (Figure 1, red markers). Additional sets of oysters were collected during the study period from Redfish Reef in Galveston Bay, from Tres Palacios Reef in Matagorda Bay, and from Panther, Chicken Foot and Middle Ground Reefs in San Antonio Bay (Figure 1, yellow markers). Results from these five reefs are referenced as supplemental reefs and included in the study evaluation. There were insufficient numbers of live oysters at four of these reefs during the initial month of the contract study period. Therefore substitute sample locations were selected in close proximity to the target monitoring locations in order to complete the contract requirements for the remainder of the study. April Fools Reef was substituted for Redfish Reef due to its mid-bay location. First Chain Island, Second Chain Island and V-Reefs were substituted for Panther, Chicken Foot and Middle Ground Reefs due to their respective proximity to the target monitoring locations. Tres Palacios Reef was monitored only one time during the summer season for supplemental information purposes and is not a substitute for the three reefs monitored in Matagorda Bay. Reef names, river basins, bay systems, and identification codes for individual reef results referenced in this study are included in Table 1.

Figure 1. Twelve primary reefs (red markers) were routinely monitored for Dermo infection by TPWD. Five supplemental reefs (yellow markers) were monitored for only one month of the study due to insufficient sample size at Redfish, Tres Palacios, Middle Ground, Panther and Chicken Foot Reefs.

Table 1. Twelve primary reefs were routinely monitored and five supplemental reefs (*) were monitored only one month of the study period. Reef names were assigned a reef code for identification in statistical analysis and graphs. River basins and bay systems where each reef is located are also included.

Reef Name	Reef Code	Bay System	River Basin/Bay System
Sabine Lake	SL1	Sabine	Sabine River/Sabine Lake
Redfish*	GB1	Galveston	Trinity River/Galveston Bay
Hanna's	GB2	Galveston	Trinity River/Galveston Bay
Fishers	GB3	Galveston	Trinity River/Galveston Bay
April Fool's	GB4	Galveston	Trinity River/Trinity Bay
Sammy's	MB1	Matagorda	Colorado River/West Matagorda
Mad Island	MB2	Matagorda	Colorado River/West Matagorda
Shell Island	MB3	Matagorda	Colorado River/West Matagorda
Tres Palacios*	MB4	Matagorda	Colorado River/West Matagorda
Gallinipper	LB2	Lavaca	Lavaca River/Lavaca Bay
Indian Point	LB3	Lavaca	Lavaca River/Lavaca Bay
First Chain	SA1	San Antonio	Guadeloupe River/San Antonio Bay
Second Chain	SA2	San Antonio	Guadeloupe River/San Antonio Bay
V-Reef	SA3	San Antonio	Guadeloupe River/San Antonio Bay
Middle Ground*	SA4	San Antonio	Guadeloupe River/San Antonio Bay
Panther*	SA5	San Antonio	Guadeloupe River/San Antonio Bay
Chicken Foot*	SA6	San Antonio	Guadeloupe River/San Antonio Bay

Sample Collection Protocols

TPWD has routinely monitored oyster populations by collecting samples once a month with a modified oyster dredge pulled for 30 seconds as per standard protocols outlined in TPWD Operations Manual (2009). During this portion of the study funded by TWDB, the sample size was to include 10 market-sized oysters ($\geq$ 76 mm), randomly selected from TPWD dredges, pulled once a month over the 12 target reefs. TPWD also collected ten oysters from the five supplemental reefs using the same Dermo sample collection protocols described for the primary target reefs.

One sample collection issue that was encountered during this study was inadequate numbers (n) of market-sized oysters collected at the target reefs. Although changing sampling locations within known Coastal Fisheries sampling grids for each reef generally resolved this problem, it did not resolve the repeated issue of inadequate numbers of live market-sized oysters collected from Hanna's Reef in lower Galveston Bay. Oysters from Hanna's Reef sustained substantial damages after Hurricane Ike in 2008. These oysters showed relatively slow recovery following the aftermath of sediments burying the oysters. No substitute reefs were available at this target monitoring location during the study. In cases where 10 market-sized oysters could not be collected each month on Hanna's Reef, samples were collected along the borders of adjacent private leases, or the number of samples evaluated was reduced for this specific target reef. Thus samples evaluated as Hanna's Reef oysters in the final evaluation are from mixed populations in Galveston Bay.

Sample Storage and Extraction Protocols

All oysters collected in the dredges were placed in burlap sacks, maintained at cool temperatures (between 10 and 20° C) using frozen plastic bottles of water, and stored in ice chests during transportation to TPWD's Dickinson Marine Laboratory and subsequently to TAMUG's Laboratory for tissue extraction. Each oyster

was opened with a sharp, sterilized oyster knife after metrics on shell length and anecdotal descriptions of meat condition and gonad condition were recorded. The anterior section of mantle tissues on both the right and left sides of each oyster were extracted using sterilized scissors and nasal forceps. Extraction utensils were sterilized by pressure cooking for 30 minutes at 140° C (37 psi) after hot soap and water washing, 70% alcohol rinse, and distilled water rinses in order to prevent cross contamination of tissue samples.

Duplicate samples (right and left mantle tissues) from each oyster were placed in separate 10-ml tubes containing 95% ethanol (QPCR assay) and fluid thioglycolate broth with 0.5-ml mixture of chloramphenocol sodium succinate and nystatin (RFTM culture). QPCR sample tubes were sterilized polyvinyl plastic vials. RFTM sample tubes were sterile glass containers. Tissues in the QPCR sample tubes were stored in a refrigerator at 4° C until DNA isolation procedures could be performed at the end of each month. Tissues in RFTM samples tubes were stored at room temperature (20° C) in a storage cabinet for seven-days under anaerobic, dark conditions before standard RFTM procedures were completed.

RFTM Methodology

The RFTM used in this study is widely used across the United States for monitoring *P. marinus* (Ray 1966). The anterior section of mantle tissues from each oyster was extracted as previously described and subsequently cultured in thioglycolate and antibiotic medium for seven-days under anaerobic, dark conditions at 20° C. Upon completion of the incubation period, the tissues were removed from tubes, placed on slides, macerated with a sterilized utensil, and the hypnospores were stained with Lugol's iodine. Each slide was examined under a light microscope for a semi-quantitative assessment (Ray 1966, Craig et al. 1989) of the numbers or density of cells present. In this study each tissue sample was examined and categorically assigned both Mackin scale disease codes (Mackin 1962) and condition ratings.

The 16 Mackin scale disease codes (Table 2) semi-quantitatively assigned for each Dermo infection category ranged from 0.00 (negative or "no *P. marinus* cells present") to 5.00 (100 % of tissues heavily infected with *P. marinus* cells). Sixteen categorical condition ratings were also qualitatively assigned for Dermo intensity that corresponded to each Mackin scale disease code (Table 2).

Table 2. RFTM categorical condition and Mackin scale disease codes assigned to each level of Dermo infection based on density of *P. marinus* cells.

Categorical Condition	Mackin scale disease codes	Description of Dermo Incidence
Negative/ No infection	0.00	no cells present
Very Light -	0.33	1-10 cells
Very Light +	0.67	11-74 cells
Light -	1.00	75-125
Light	1.33	>125 cells but < 25% of tissues is cells
Light +	1.67	<25% of tissues is cells
Light/moderate -	2.00	25% of tissues is cells
Light/moderate	2.33	>25% but much < 50% of tissues is cells
Light/moderate +	2.67	>25% but < 50% of tissues is cells
Moderate -	3.00	50% of tissue is cells
Moderate	3.33	>50% but much < 75% of tissues is cells
Moderate +	3.67	>50% but < 75% of tissues is cells
Moderately Heavy -	4.00	75% of tissues is cells
Moderately Heavy	4.33	>75% but much less than 100% of tissues is cells
Moderately Heavy +	4.67	>75% of tissue is cells, oyster tissue still visible
Heavy	5.00	100% of oyster tissue covered by cells

QPCR Methodology

Prior to DNA isolation, tissue samples were removed from the vials and placed on clean paper towels to begin the genomic DNA isolation process. Each tissue sample was macerated with sterile scissors and forceps, and approximately 50 mg of this macerated tissue was subsampled and placed in 2.0-ml sterile vials. Genomic DNA was isolated from these subsamples using a Puregene® miniprep kit (Gentra Systems, Minneapolis, MN, U.S.A.). Manufacturer's instructions were followed with slight modifications that are documented in Appendix A. Following the DNA purification procedure, sample DNA was eluted in 100 or 250 μl of DNA rehydration solution (provided with kit). The amount of elution was dependent on pellet size. Samples were then transported to the Perry R. Bass Marine Research Laboratory (PRBMFRS) (see Figure 1) for QPCR analysis. The initial DNA concentration of each sample was estimated by measuring A260 nm absorbance on a spectrophotometer and comparing this to undigested λ-phage DNA of known concentration. Dilution factors were calculated from the λ-phage standard, and the DNA concentration for experimental samples was adjusted to 50 ng/μl by dilution with an appropriate amount of water.

As previously stated, the Gauthier et al. (2006) methodology (QPCR) was chosen for this study, due to its apparent repeatability as well as its exclusivity to *P. marinus*. The Gauthier et al. (2006) methodology uses the mechanics of QPCR to measure the amount of DNA present by amplifying a small targeted segment of the internal transcribed spacer (ITS) locus, called a Perk-1 locus. Theoretically, increased presence of *P. marinus* cells (higher levels of parasitemia) result in increased volume of parasite DNA per gram of oyster tissue. A labeled DNA probe specific to the Perk-1 locus fluoresces in direct correlation with the amount of double-stranded target DNA available after each cycle of the reaction, and florescence is exponentially distributed over time. Thus larger amounts of target DNA (more parasitic cells present initially) will amplify rapidly, and fluoresce at a faster rate than a sample with fewer target molecules present initially. The quantitative end product of QPCR is the threshold cycle time (Ct), which is the cycle at which the slope of the measureable fluorescence becomes greater than 1.0 from one cycle to the next. Thus heavier infections will result in an earlier reaction time (i.e., smaller Ct). As a result, Ct will be inversely correlated with the amount of parasite cells present.

In this study, the Gauthier et al. (2006) methodology was used with minor modifications tailored to the capabilities of PRBMFRS. Briefly, a Taqman® MGB probe (Applied Biosystems, Foster City, CA) was designed along with forward and reverse primers corresponding to the Perk-1 region as described previously (Gauthier et al. 2006). Reactions were carried out in 20 μl volumes, with the following constituents: 2 μl of template DNA (100 μM), 8 μl of 2.5x RealMaster Mix (Eppendorf, Westbury, NY), 1 μl of 20x enhancer solution (Eppendorf), 8 μl of ultrapure water and 1 μl of a mixture of both primers (18 μM, forward and reverse) and probe (5 μM). The cycling regime was identical to that of Gauthier et al. (2006), and cycling was carried out on an Eppendorf Mastercycler® ep realplex4 thermocycler.

Comparison Methodologies

Comparisons between RFTM (Mackin scale disease codes) and QPCR (Ct) results were assessed based on 1,688 samples collected in five bay systems from 12 primary target reefs and five supplemental reefs. SPSS 17.01 (SPSS 2010) statistical software (http://www.brothersoft.com/spss-268831.html) was used to compute descriptive statistics, correlations for individual results for each set of data using Pearson's Correlation Coefficient, least squares linear regression, and box plots. Probability plots of the all samples using both methodologies were also evaluated for normality of the data. In this study RFTM (Mackin scale disease codes) categories were considered the independent variable for each sample, whereas QPCR (Ct) values were considered the response variable for each sample.

Least squares linear regression was utilized to evaluate the individual RFTM (Mackin scale disease codes) and QPCR (Ct) results for each bay system separately to determine potential spatial components of the data.

In addition, an analysis of covariance (ANCOVA) was used to evaluate the slopes of RFTM (Mackin scale disease codes) categories and QPCR (Ct) values for each bay system. Box's Test of Equality of Covariance Matrices was used to test the null hypothesis: the observed covariance matrices of the dependent variables (RFTM and QPCR) are equal across all five bay systems. Box plots were utilized to assess individual RFTM (Mackin scale disease codes) and QPCR (Ct) results for each reef separately to determine potential spatial components of the data. These additional tests were conducted in order to assess the potential for reducing the number of reefs sampled by targeting specific sentinel reefs in each bay system.

Additional comparisons between RFTM (Mackin scale disease codes) and QPCR (Ct) results were evaluated for four separate seasons using box plots and frequency plots to determine potential temporal components of the data. These additional tests were conducted in order to assess the potential for reducing sample collection to specific seasons in lieu of a monthly monitoring program.

RESULTS

Sensitivity Testing

The QPCR assay used in this study is highly sensitive to variability in the amount of parasite DNA present. In a pilot study conducted in 2007 (TPWD unpublished data), the sensitivity of the QPCR assay in detecting different quantities of *Perkinsus* DNA of "Dermo-positive" oyster tissues were subjected to the QPCR methodology. Two independent oyster samples (a) and (b) were collected for the purpose of testing serial dilutions of the target DNA. These two samples tested Dermo-positive in an initial (non-quantitative) PCR test, and were indicative of moderate infection. Following this test, each sample was diluted 1:10, 1:100 and 1:1000. Undiluted samples and serial dilutions were run together on the same plate, and the sensitivity of QPCR was assessed by regression of dilution level with threshold cycle reaction times (Ct) for two independent oysters (a) and (b). The results shown in Figure 2 show a very clear relationship between dilution factor and threshold cycle reaction time (Ct) with an $r^2 > 0.99$ in each case. These results suggest that variability between samples will be highly predictive of the quantity of template DNA available to the reaction, assuming relatively low pipette error or heterogeneity in template DNA purity.

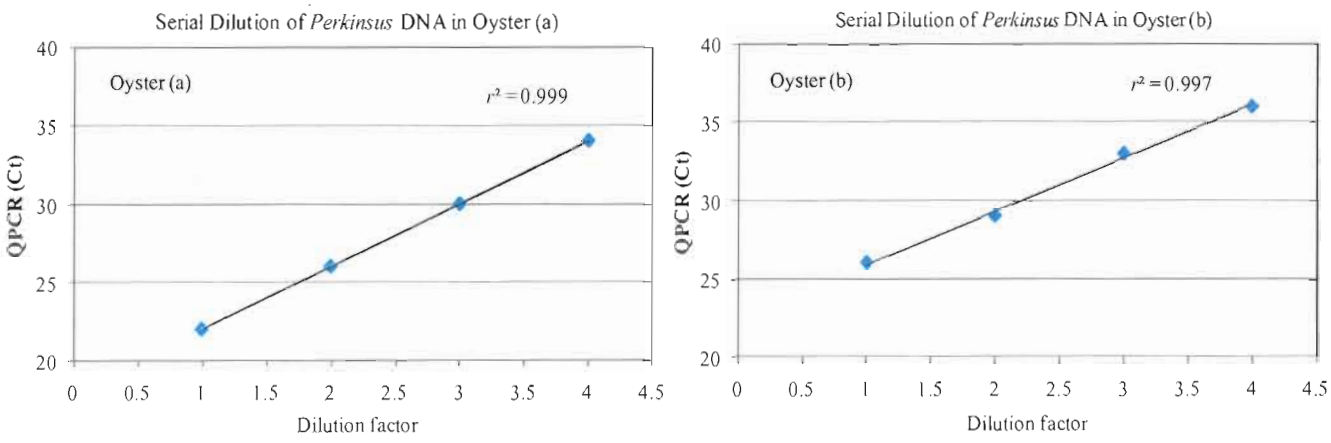

Figure 2. Serial dilution factors using QPCR methodology to determine threshold cycle reaction times (Ct) of target DNA in two independent oysters (a) and (b) in a 2007 pilot study (TPWD unpublished data).

All Sample Comparisons

In this study, QPCR (Ct) results based upon the initial quantity of Dermo DNA template present with heavier infections resulted in an earlier reaction time (smaller Ct value of 12-20 Ct) and Dermo DNA template present with light infections resulted in longer reaction times (larger Ct value of 25-40 Ct). When no Dermo DNA template was present the reaction time was recorded as the maximum reaction time (40 Ct). Thus, QPCR (Ct) values were inversely correlated with the amount of parasite cells present, and were inversely related to RFTM (Mackin scale disease codes) categorical values.

The results of QPCR were compared to the results of the RFTM for all samples and divided into four categories (Table 3). The first category represents 38.03 percent (n=642) of the oysters that tested positive for various levels of infection using the RFTM, and was verified by positive QPCR (Ct) results. The second category represents 40.58 percent (n=685) of the oysters that tested negative and "no *P. marinus* cells present" using the RFTM in contrast to these same oysters testing negative for infection by QPCR.

The third category represents 17.65 percent (n=298) of the oysters tested negative for RFTM or were diagnosed as "no *P. marinus* cells present" in contrast to these same oysters testing positive for infection by QPCR. In this third category, 64.50 percent (n=195) of these negative RFTM (Mackin scale disease codes) results also occurred when water temperatures were low (< 10 °C) during the winter and the early spring months of 2009-2010 of this study. These negative RFTM (Mackin scale disease codes) results are similar to findings reported by Gauthier et al. (2006), which suggested that QPCR is more sensitive to light infection intensity than RFTM. These results also indicate that QPCR may be more sensitive to detecting some level of infection present in the oyster during colder temperatures when *P. marinus* is not detected using RFTM (Chu and Greene 1989).

The fourth category represents 3.74 percent of the oysters (n=63 samples) that tested positive using the RFTM but tested negative using the QPCR. Positive RFTM (Mackin scale disease codes) results in this fourth category may be due to a variety of reasons that have not yet been fully evaluated in the current study. There could be loss of parasite cells during the genomic DNA isolation procedures because a smaller portion of the mantle tissue is evaluated using QPCR procedures than RFTM uses. *P. marinus* cells tend to concentrate in the veins of the anterior mantle tissues near the hinge hence the preference for choosing this location for tissue extraction to determine Dermo infection in each oyster. However, low numbers of *P. marinus* cells may not be equally distributed in the anterior portion of the mantle tissues when the mantle veins are not equally distributed throughout the mantle tissues. Unequal distribution of the mantle veins often occurs during the later stages of gonadal development when excess gametes extend into the mantle tissues so as to obscure the veins and potentially could alter the distribution of *P. marinus* cells extracted for duplicate samples. In this study, mantle veins were not easily observed during this later stage of gonadal development. In contrast, mantle veins were easily observed after the gonads were depleted so all the tissues appeared watery and translucent. The number of veins on each side of the mantle tissues was recorded during one month of this study in order to further evaluate any differences that may have contributed to positive RFTM (Mackin scale disease codes) categories when QPCR (Ct) values were negative. The results of that test are not included in this report due to the short time frame of this study.

Table 3. Presence or absence of Dermo infection by both RFTM and QPCR methodologies (n=1,688).

Category	n	% of Total
Both Positive (RFTM + PCR+)	642	38.03
Both Negative (RFTM - PCR-)	685	40.58
RFTM Negative (-) QPCR Positive (+)	298	17.65
RFTM Positive (+) QPCR Negative(-)	63	3.74

Correlation Analysis of All Samples

Pearson Correlation Coefficients for all samples that were parallel tested using RFTM and QPCR methodologies to obtain Dermo infection are shown in Table 4. The results demonstrated there is a significant correlation between RFTM (Mackin scale disease codes) categorical data and QPCR (Ct) continuous data ($P < 0.0001$). QPCR (Ct) results were also negatively correlated with salinity and temperature, whereas RFTM (Mackin scale disease codes) results were positively correlated with salinity and temperature. These results are consistent with the methodology used and the intensity of Dermo's response to environmental variables. Summary statistics for these correlations are shown in Appendix B, Tables B1 - B6.

Table 4. RFTM and QPCR correlation matrix with environmental variables: salinity and temperature.
Pearson's Correlation Coefficient (r), N= 1,688 Probability > |r| under H0: r = 0

	RFTM	QPRC	Salinity	Temperature
RFTM	1.000	-0.862	0.256	0.235
Significance		<0.001	<0.001	<0.001
QPCR	-0.862	1.000	-0.218	-0.147
Significance	<0.001		<0.0001	<0.001
Salinity	0.256	-0.218	1.000	0.312
Significance	<0.001	<0.0001		<0.001
Temperature	0.235	-0.147	0.312	1.000
Significance	<0.001	<0.001	<0.001	

Linear Regression of All Samples

Collective results from all 1,688 samples parallel tested in this study demonstrated a linear inverse relationship between RFTM (Mackin scale disease codes) categorical data and QPCR (Ct) continuous data, with linear regression equation QPCR = -5.407*(RFTM) + 38.018 and correlation coefficient $r^2 = 0.743$ ($P < 0.001$, Figure 3). This figure also shows individual QPCR (Ct) values have a broad range of corresponding upper and lower 95% confidence intervals (CI) around the mean regression line. The greatest variation of individual QPCR (Ct) values occurred at the 0.00 and 0.67 Mackin scale disease codes.

Seven major categorical levels of infection (no infection, very light, light, light-moderate, moderate to moderately-heavy, and heavy) are shown in Figure 3 to demonstrate the broad range of respective RFTM (Mackin scale disease codes) that can be categorically assigned to individual QPCR (Ct) values. These results indicate that compiling all samples from different bay systems and from different reefs may not be a suitable methodology for predicting or assigning a specific RFTM (Mackin scale disease code) category to an individual QPCR (Ct) value.

The Mackin scale disease code categories are reported on http://www.oystersentinel.org/ website as mean weighted prevalence values based on the average of the Mackin scale disease codes in 10 oysters sampled at each reef site during one monthly sampling effort because individual results are not representative of the average condition of the population being tested at that one point in time. Based on this principle, the QPCR (Ct) values associated with each replicate RFTM sample value in this study were averaged to determine the most probable QPCR (Ct) value that could be assigned to a specific RFTM (Mackin scale disease code) category (Table 5). Consequently, the average QPCR values derived from all samples from all bays and all reefs have a much narrower range of upper and lower 95% confidence intervals with linear equation QPCR = -4.891* (RFTM) + 38.580 and correlation coefficient $r^2 = 0.807$ ($P < 0.001$). Thus, average QPCR (Ct) values appear more representative of the average Dermo infection level in the population of oysters being tested.

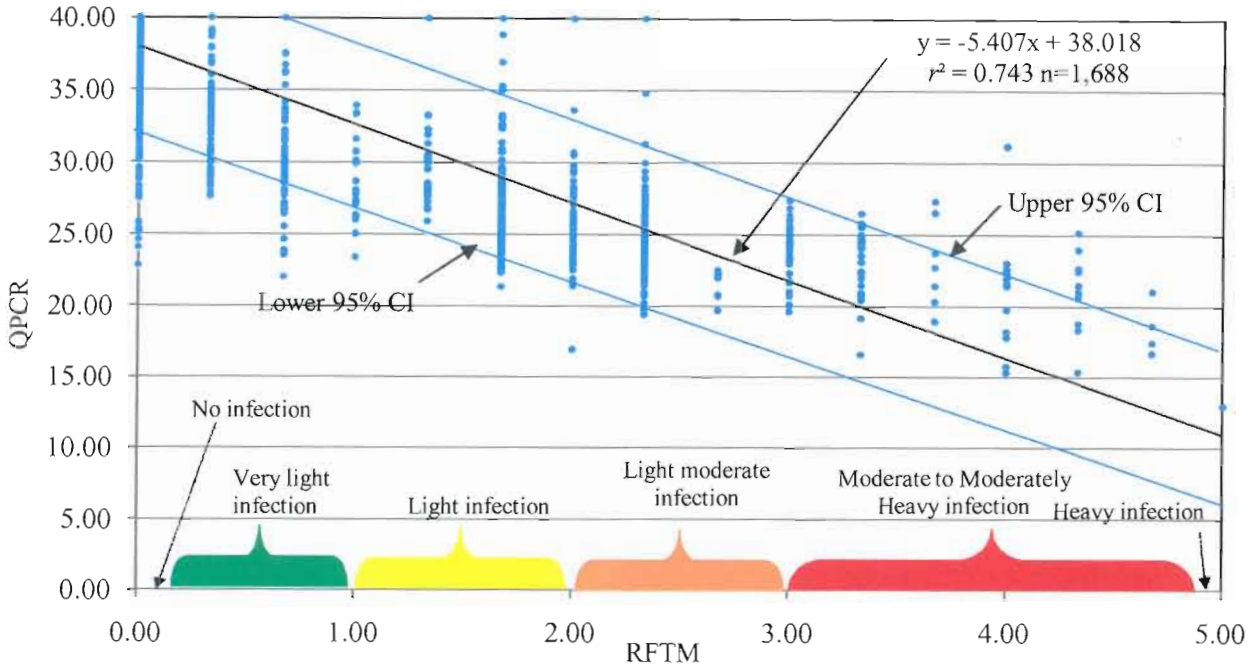

Figure 3. Linear regression of all individual QPCR (Ct) values correlated with replicate RFTM (Mackin scale disease codes) categorical values, and individual upper and lower 95% confidence intervals (CI).

Table 5. Average QPCR (Ct) values with upper and lower confidence interval (CI) assigned to each RFTM (Mackin scale disease codes) based on linear regression equation QPCR= -4.891*(RFTM)+38.580 r^2=0.807.

RFTM	Avg QPCR	95% Lower CI	95% Upper CI	No. Samples
0.00	38.58	38.40	38.75	983
0.33	35.94	35.16	36.71	105
0.67	31.24	30.19	32.29	77
1.00	28.50	27.55	29.45	28
1.33	29.82	28.55	31.08	28
1.67	27.44	26.86	28.02	151
2.00	25.62	25.01	26.23	85
2.33	24.99	24.42	25.55	128
2.67	21.42	20.77	22.07	9
3.00	23.51	22.82	24.20	33
3.33	22.60	21.70	23.49	25
3.67	22.96	20.66	25.26	7
4.00	20.93	18.76	23.10	13
4.33	21.01	19.39	22.63	11
4.67	18.45	16.59	20.32	4
5.00	12.98	12.96	13.00	1

Box Plots of All Samples

Comparison of all samples parallel tested in this study presented in box plot (Figure 4) showed median, upper and lower quartiles of the individual QPCR (Ct) values for each RFTM (Mackin scale disease code) category. These box plots demonstrated individual QPCR (Ct) values have a broad range of values (40.00 to 12.00 Ct) corresponding to each RFTM (0.00 to 5.00 Mackin scale disease codes) category. These results verify QPCR produced the broadest range of (40.00 to 27.00 Ct) values which corresponded to 0.33 to 0.66

Mackin scale disease codes (very low levels of infection) observed for parallel tested samples assayed by RFTM. QPCR also produced the narrowest range of (26.00 to 22.00 Ct) values which corresponded to 2.00 to 2.67 Mackin scale disease codes (light/moderate levels of infection) observed for parallel tested samples assayed by RFTM.

Only one QPCR result (12.00 Ct) was observed in all the samples from five bay systems. This same sample was observed to also have the highest infection level (5.00 Mackin scale disease code) detected by RFTM in all the samples from five bay systems. In addition, very few individual oysters with high levels of infection (4.00 to 5.00 Mackin scale disease codes) were detected by RFTM. Both methodologies showed there were few samples with high levels of infection. These results indicate there may be less "intense" infection in all the populations sampled, or there may be fewer oysters surviving at the highest lethal level of Dermo infection and therefore less likely to be randomly collected by the dredge samples on each reef.

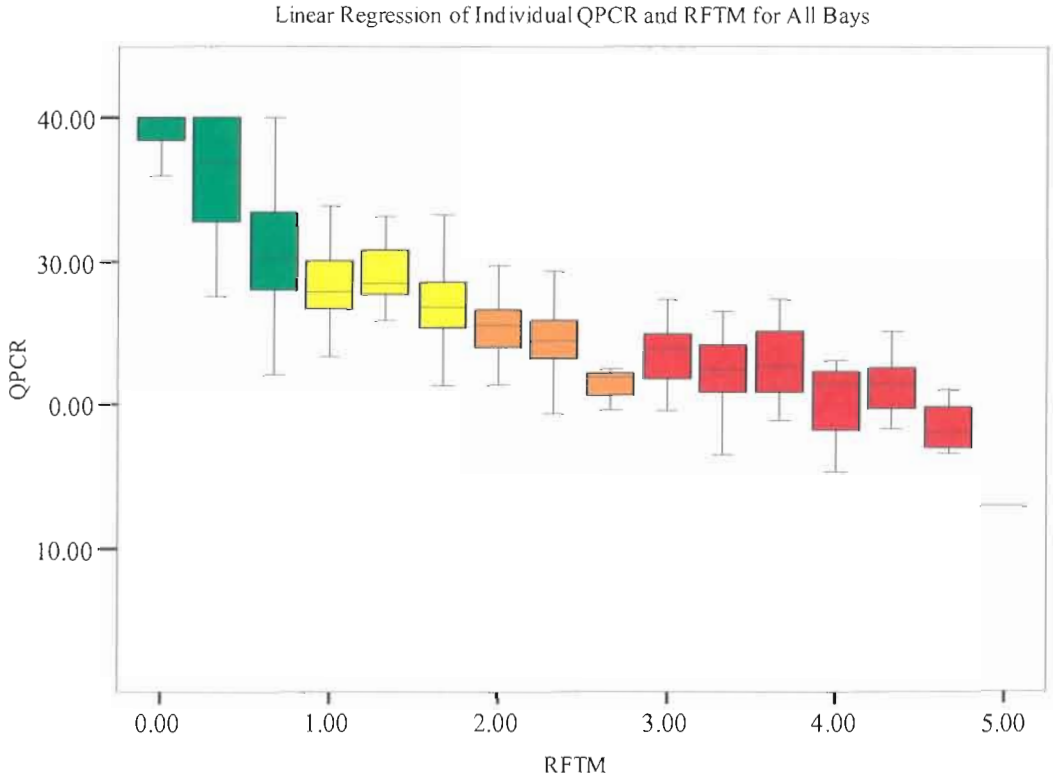

Figure 4. Box plots of all samples showing median, upper and lower quartiles of the individual QPCR (Ct) values for each RFTM (Mackin scale disease code) category. Color indicates condition category for increasing infection levels (green - very light, yellow - light, orange - light to moderate, red -moderate to moderately heavy). The single horizontal line at RFTM - 5.0 represents the only sample collected at heavy infection condition category.

Normality Test of All Samples

The probability plot of the standardized residuals for all samples assayed using RFTM (Figure 5) and QPCR (Figure 6) methodologies shows the data is not normally distributed. These probability plots for all samples show there was a significant variation of both RFTM (Mackin scale disease codes) and QPCR (Ct) values at both the lowest and highest levels of Dermo infection for all samples assayed. The 16 Mackin scale codes shown in Figure 5 are not continuous data in contrast to QPCR (Ct) continuous data shown in Figure 6. The data was not transformed to normalize the distributions in this study for easier conversion of predicted values into actual values for each methodology.

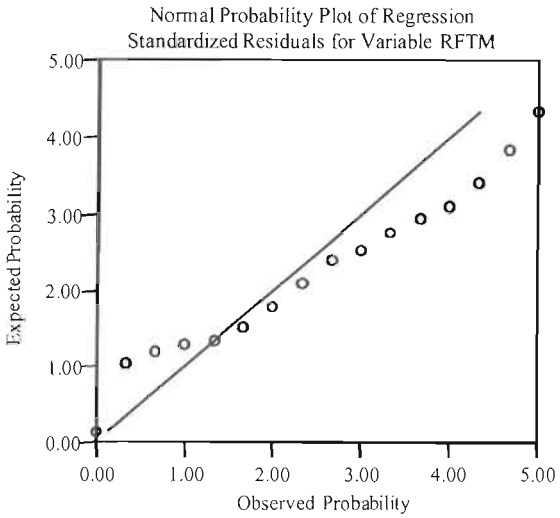

Figure 5. Probability plot of standardized residuals for all samples tested by RFTM.

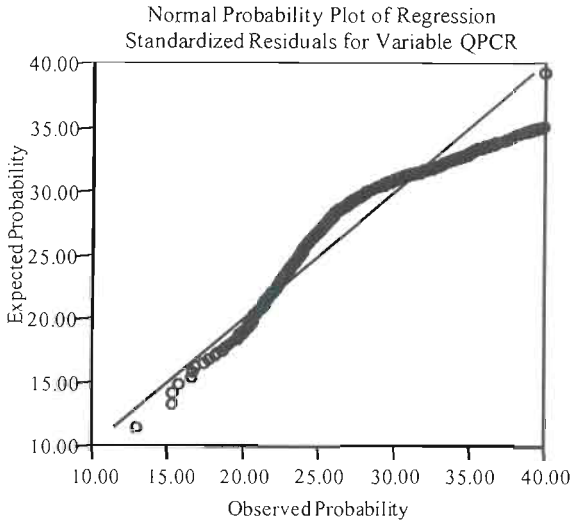

Figure 6. Probability plot of standardized residuals for all samples tested by QPCR.

Sabine Lake Comparisons

Individual sample results from Sabine Lake's one oyster reef (n = 140) demonstrated an inverse linear relationship between RFTM (Mackin scale disease codes) and QPCR (Ct) values (Figure 7), with least squares linear regression equation QPCR = -3.779*(RFTM) + 35.346 and a correlation coefficient of r^2 = 0.538 ($P < 0.001$). Summary statistics for RFTM (Mackin scale disease codes) and QPCR (Ct) correlations are shown in Appendix B, Tables B7 and B8. Sabine oysters often smelled of hydrocarbons during tissue extraction procedures. Mantle tissues from Sabine Lake were watery and the oysters were predominantly emaciated during the majority of the study period regardless of salinity conditions. Watery or degraded tissues from this reef made duplicate extraction procedures more difficult to accomplish.

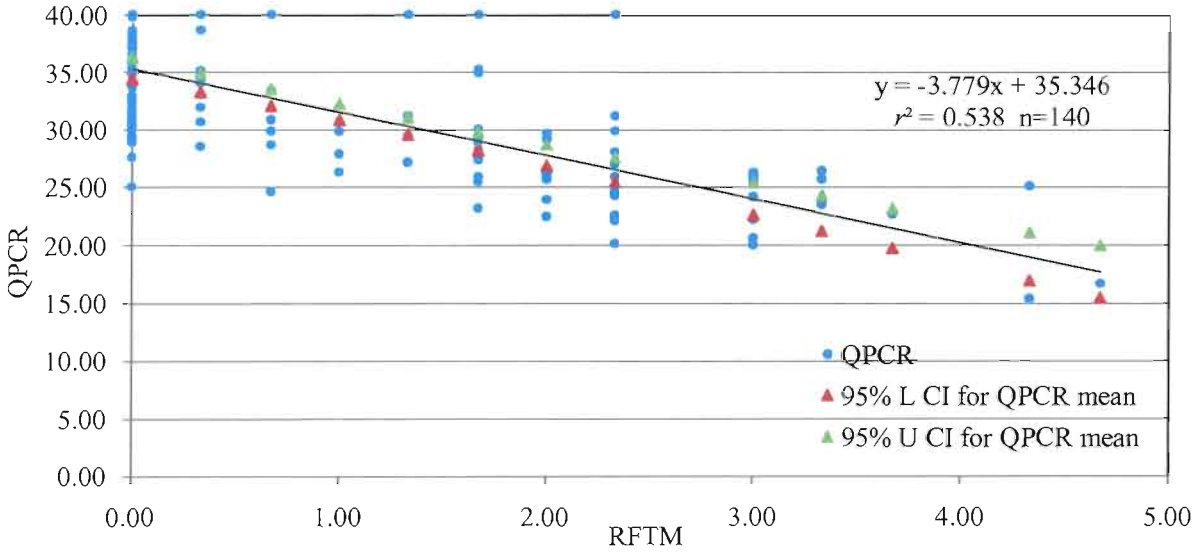

Figure 7. Scatter plot of Sabine Lake samples showing trend line for individual QPCR (Ct) and RFTM (Mackin scale disease codes), with upper and lower 95% confidence intervals (CI).

Galveston Comparisons

Sample results from Galveston Bay samples (n = 389) collected at Fishers Reef (upper Trinity Bay), April Fools Reef (mid-bay), Hanna's Reef (lower Galveston Bay) and one set of ten samples from Redfish Reef demonstrated an inverse relationship between RFTM (Mackin scale disease codes) and QPCR (Ct) values (Figure 8), with least squares linear regression equation QPCR = -5.642*(RFTM) + 38.494 and a correlation coefficient of $r^2 = 0.738$ ($P < 0.001$). Summary statistics for RFTM (Mackin scale disease codes) and QPCR (Ct) correlations are shown respectively in Appendix B, Tables B9 and B10. Tissue samples from all reefs evaluated in Galveston Bay were healthy and there were no problems with extracting duplicate samples for evaluation of the two methodologies. Sample results covered the full range of "not infected" to "heavily infected" oysters in Galveston Bay. Results were comparable with historic trends for these same reefs.

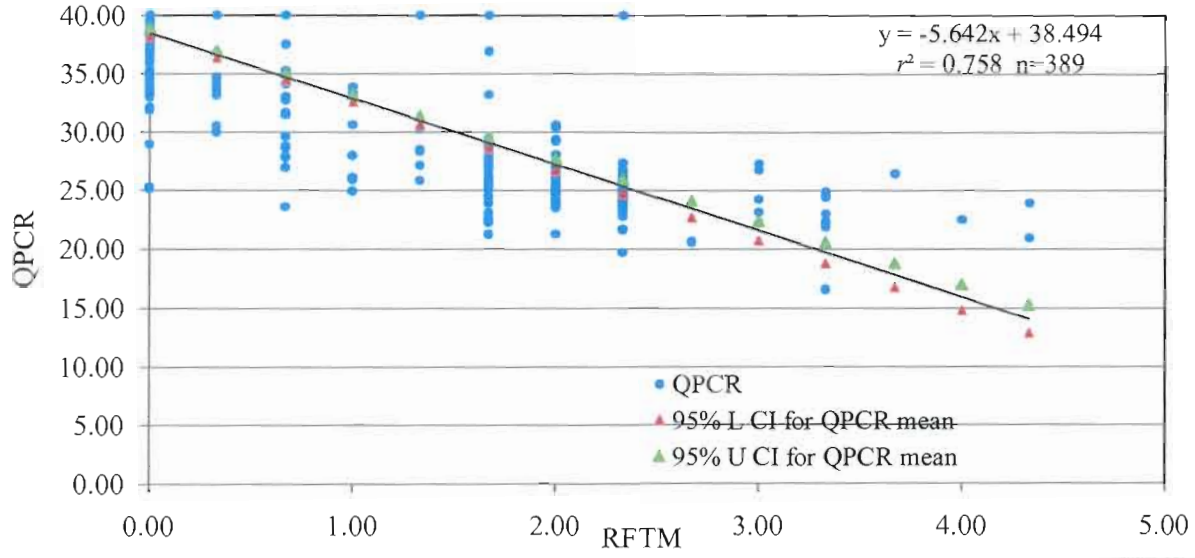

Figure 8. Scatter plot of Galveston Bay samples showing trend line for individual QPCR (Ct) and RFTM (Mackin scale disease codes), with upper and lower 95% confidence intervals (CI).

Matagorda Bay Comparisons

Sample results from Matagorda Bay samples (n= 429) collected at Shell Island Reef (closest to Colorado River), Mad Island Reef (mid-bay), Sammy's Reef (farthest from Colorado River) and one set of ten samples from Tres Palacios Bay Reef demonstrated an inverse relationship between RFTM (Mackin scale disease codes) and QPCR (Ct) values (Figure 9), with least squares linear regression equation QPCR = -4.976*(RFTM) + 37.070 and a correlation coefficient of $r^2 = 0.751$ ($P < 0.001$). Summary statistics for RFTM (Mackin scale disease codes) and QPCR (Ct) correlations are shown respectively in Appendix B, Tables B11 and B12. Tissue samples from all three reefs evaluated in Matagorda Bay were healthy and there were no problems with extracting duplicate samples for evaluation of the two methodologies. Sample results covered the full range of "not infected" to "heavily infected" oysters in Matagorda Bay. No infected cells (0.0 - Mackin scale disease codes) were present in Shell Island Reef samples during the entire study period. This reef is located geographically closer to the Colorado River, and historically has been reported to have very low to no Dermo infection (0 to 1.0 - Mackin scale disease codes). Mad Island and Sammy's Reef samples, located further from the Colorado River have historically have been reported to have low to high levels of infection (1.67 to 4.0 Mackin scale disease codes) depending on precipitation or inflows from the Colorado River. Results from all three reefs in this study are comparable with historic trends for these same reefs.

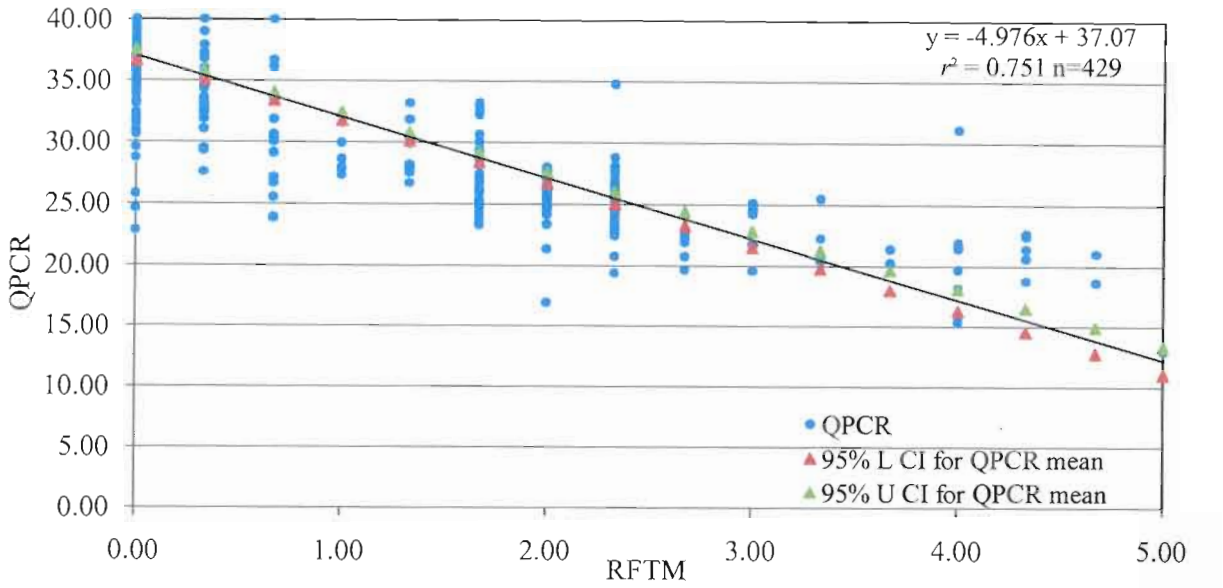

Figure 9. Scatter plot of Matagorda Bay samples showing trend line for individual QPCR (Ct) and RFTM (Mackin scale disease codes), with upper and lower 95% confidence intervals (CI).

Lavaca Bay Comparisons

Sample results from Lavaca Bay samples (n = 280) collected at Gallinipper Reef (closest to Lavaca River) and Indian Point Reef (farthest from Lavaca River) demonstrated an inverse relationship between RFTM (Mackin scale disease codes) and QPCR (Ct) values (Figure 10), with least squares linear regression equation QPCR = -4.867*(RFTM) + 36.077 and a correlation coefficient of r^2 = 0.661 (P < 0.001). Summary statistics for RFTM (Mackin scale disease codes) and QPCR (Ct) correlations are shown respectively in Appendix B, Tables B13 and B14. Tissue samples from both reefs evaluated in Lavaca Bay were healthy and there were no problems with extracting duplicate samples for evaluation of the two methodologies. Sample results covered the full range of "not infected" to "heavily infected" oysters in Lavaca Bay. Results from these two reefs in this study are comparable with historic trends for these same reefs.

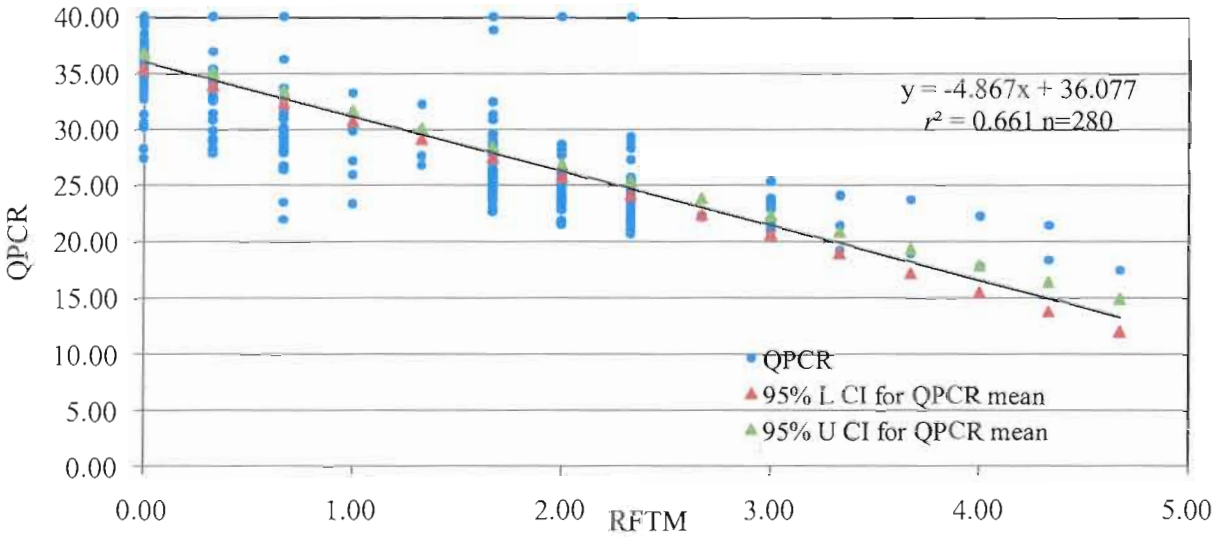

Figure 10. Scatter plot of Lavaca Bay samples showing trend line for individual QPCR (Ct) and RFTM (Mackin scale disease codes), with upper and lower 95% confidence intervals (CI).

San Antonio Bay Comparisons

Sample results from San Antonio Bay samples (n = 449) collected at V-Reef (closest to Guadalupe River), Second Chain Island Reef (western area of bay), First Chain Island Reef (farthest from Guadalupe River and closest to Pass Cavillo) in addition to three sets of ten oyster samples from Panther Reef, Chicken Foot Reef, and Middle Ground Reef (October 2009) demonstrated an inverse relationship between RFTM (Mackin scale disease codes) and QPCR (Ct) values (Figure 11), with least squares linear regression equation QPCR = -5.405* (RFTM) +39.337 and a correlation coefficient of $r^2 = 0.6147$ ($p < 0.0001$). Summary statistics for RFTM (Mackin scale disease codes) and QPCR (Ct) correlations are shown respectively in Appendix B, Tables B15 and B16.

Tissue samples from San Antonio Bay reefs were watery or degraded for the majority of the study period. The quality of tissues created additional problems for extracting equal or adequate mantle tissues to be evaluated by both methodologies in this study. V-Reef oysters historically have had no level of infection or very low levels of Dermo infection since 2003. Results from V-Reef in this study were consistent with historic trends. RFTM sample results for the other five reefs sampled only covered half the historic range of Dermo infection levels previously recorded for San Antonio Bay. Results from San Antonio Bay in general had low levels of Dermo (< 1.67 Mackin scale disease codes) using RFTM throughout the study period. In contrast genomic DNA for Dermo infection using QPCR detected higher levels of infection in the samples for the majority of study period including winter samples during record low temperatures. Towards the end of the study period (October 12, 2010) oysters from Second Chain Island and V-Reef samples were observed to be partially frozen when they were opened for tissue extraction at the TAMUG Lab. First Chain Island samples from this same October 12, 2011 collection period were not visibly frozen. Further investigation revealed that samples up to this date may have become frozen while temporarily stored in a refrigerator. The potential for this occurrence was corrected for the remainder of the study.

A second sampling effort of these same three reefs was initiated on October 18, 2010 and live samples were processed immediately (without prior storage in a refrigerator). First Chain Island RFTM (Mackin scale disease codes) results for both October sampling efforts were generally comparable to levels of infection detected by QPCR for this reef. However, Second Chain Island RFTM (Mackin scale disease codes) results from the two October sampling dates showed there were marked differences between previously collected tissues versus tissues collected and processed immediately. The RFTM (Mackin scale disease codes) results from Second Chain Island on the first October sample date showed very low levels of Dermo infection (0.33 Mackin scale disease codes) to no detectable levels of Dermo infection (0.0 Mackin scale disease codes). The RFTM (Mackin scale disease codes) results from Second Chain Island on the second October sample date (only a week later) showed moderate to high levels of Dermo infection (1.0 to 2.0 Mackin scale disease codes). The results from Second Chain Island on both October sampling dates showed moderate levels of Dermo infection by QPCR (27 to 24 Ct). Results for V-Reef samples exhibited no detectible levels of Dermo infection by RFTM or QPCR methodologies and were consistent with prior results as well as historic trends for this upper bay reef. Therefore V-Reef samples were not compared for differences in detection levels of the two methodologies using frozen and non-frozen samples. However, the results of Second Chain Island sample comparison suggest that freezing the oyster tissues may degrade the tissues so it is no longer considered a viable culture tissue for evaluation of *P. marinus* by RFTM.

Although freezing the tissues causes difficulties with extraction of the mantle, the genetic material appears to remain intact for evaluation of *P. marinus* by QPCR. In addition QPCR based assays in this study proved to be more sensitive than RFTM assays in detecting *P. marinus* in frozen tissues. The number of San Antonio Bay samples available for comparison between frozen and not frozen tissues from these two sample dates in October is not adequate to speculate further, or to eliminate any previous results for comparison of these two methodologies. However, no significant differences were observed in the October 2010 results for other bay systems comparing both methodologies.

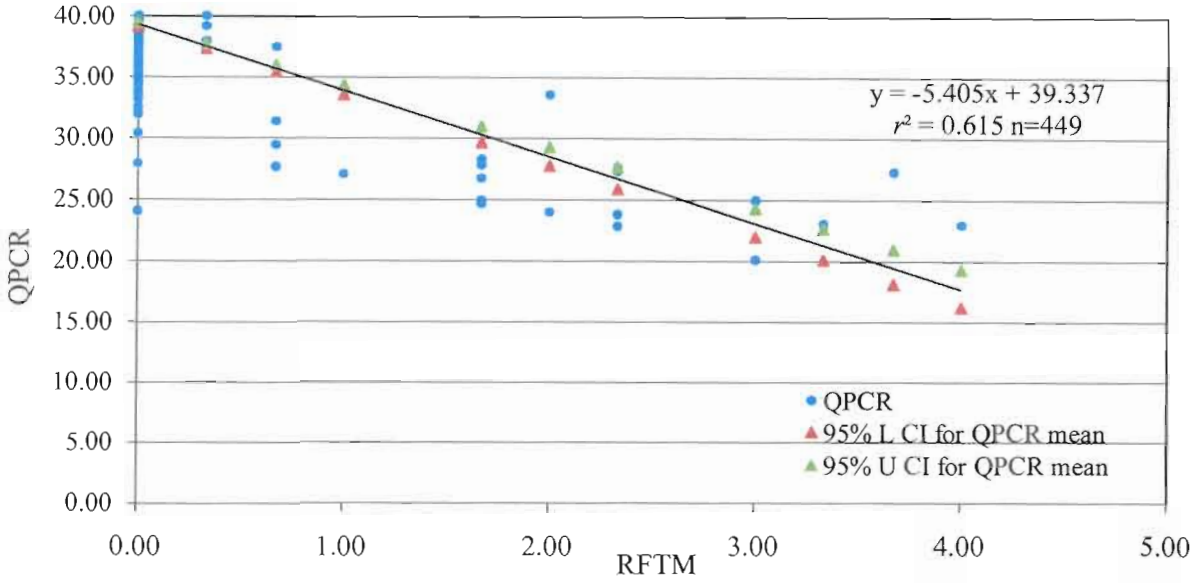

Figure 11. Scatter plot of San Antonio samples showing trend line for individual QPCR (Ct) and RFTM (Mackin scale disease codes), with upper and lower 95% confidence intervals (CI).

Individual Bay Differences

An evaluation of the least squares linear regression models for each of the five bay systems (Figures 7 through 11) indicates the slopes and intercepts are not equal across bay systems. The least variation for all five graphs occurs at approximately the RFTM (Mackin scale disease code) category of 2.00, which is also the threshold value for intense progression of the disease that begins to interfere with the oyster's physiological condition, inhibits spawning, growth and ultimately contributes to the death of the individual oyster. The least variation for QPCR (Ct) values for all five regression lines occurs between 25 and 27 Ct which also corresponds to RFTM (Mackin scale disease code) category of 2.00 and a qualitative condition category of "light moderate infection, as previously defined in Table 2.

An analysis of covariance (ANCOVA) was used to compare slopes of RFTM (Mackin scale disease codes) categories and QPCR (Ct) values for each bay system for spatial components. Results show the slopes are not equal and there are significant differences between bay systems and within bay systems (Table 6).

Table 6. Analysis of covariance for RFTM (Mackin scale disease codes) and QPCR (Ct) values between and within bay systems.

Variables	Covariate:Bays	Sum of Squares	df	Mean Square	F	Significance
RFTM	Between Bays	297.46	4	74.37	75.89	0.00
RFTM	Within Bays	1,649.08	1,683	0.98		
RFTM	Total	1,946.54	1,687			
QPCR	Between Bays	17,422.95	4	4,355.74	123.82	0.00
QPCR	Within Bays	59,205.96	1,683	35.18		
QPCR	Total	76,628.91	1,687			

Box's Test of Equality of Covariance Matrices was used to test the null hypothesis: the observed covariance matrices of the dependent variables (RFTM and QPCR) are equal across all five bay systems. Test results (Box's M) show there are significant differences ($P < 0.001$) between bay systems and the variances of the

dependent variables (RFTM and QPCR) in each bay system are not homogenous or equal across groups (Table 7). These differences between bay systems indicate that each bay system may need to be evaluated separately in future monitoring programs.

Table 7. Box's Test of Equality of Covariance Matrices (M) for RFTM (Mackin scale disease codes) and QPCR (Ct) for the five bay systems in this study.

Box's M	F	df^1	df^2	Significance
594.60	49.39	12	3,716,803	0.00

Individual Reef Differences

The range of infection at individual reefs varied in all bays. Oysters from reefs that were located closer to freshwater sources that have historically had no detectable Dermo infection continued to maintain these same profiles throughout the study period. Oyster from reefs that were located further away from freshwater sources that have historically been reported to have moderate to heavy Dermo infection had similar trends in this study with the exception of San Antonio Bay oysters.

Descriptive statistics for individual reefs are shown in Appendix B, Tables B17 and B18. No *P. marinus* cells (Mackin scale disease code - 0.00) resulting in the "no infection" or negative condition category were assigned by RFTM for oysters collected from Fishers Reef in Galveston Bay (Appendix B, Table B17). In contrast, duplicate tissue samples from the same oysters collected at Fishers Reef evaluated by QPCR were not consistently negative and measured 31 - 39 Ct values corresponding to the "very light (- and +)" infection condition category (RFTM) over the study period (Appendix B, Table B18). No other reef evaluated during this study period was found to consistently test "negative" or "no infection" condition category (RFTM) with no *P. marinus* cells (Mackin scale disease code - 0.00) detected.

Differences between reefs were evaluated using box plots to show median and upper and lower quartile ranges for samples from each reef using RFTM (Figure 12) and QPCR (Figure 13). Median values of sample results indicated oysters collected on reefs located near freshwater sources (Fishers Reef (GB3) in Trinity/ upper Galveston Bay and Shell Island Reef (MB3) in Matagorda Bay) measured "no *P. marinus* cells" (0.00 Mackin scale disease code) or the "very light" infection condition category (0.33-0.67 Mackin disease code) by RFTM. These results corresponded to 40 to 37 Ct values measured using QPCR (Figure 13) on duplicate samples from these same reefs during all months of the study period. Historic trends for fresher conditions at Fishers and Shell Island Reefs may have some influence over these populations of oysters that typically have less Dermo infection than other reefs in Galveston or Matagorda Bays.

The box plot median values of sample results using RFTM (Figure 12) showed reefs located further away from freshwater sources and influenced by Gulf of Mexico salinities (Sabine (SL1), April Fools (GB4), Hanna's (GB2), Sammy's (MB1), Mad Island (MB2), Tres Palacios (TRP1), Gallinipper (LB2), and Indian Point Reefs (LB3)) measured relatively "moderate" condition category levels of Dermo infection (1.00 to 2.00 Mackin disease codes) during all months of the study period. These results corresponded to 37 to 27 Ct values measured using QPCR (Figure 13) on duplicate samples from these same reefs during all months of the study period.

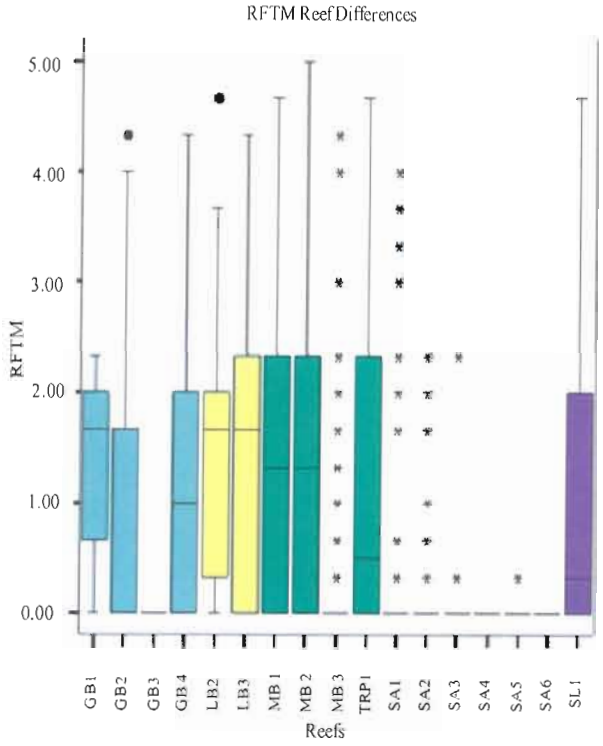

Figure 12. Box plot showing median, upper and lower quartiles for RFTM (Mackin scale disease codes) categories at individual reefs. Reef codes for twelve primary reefs and five supplemental reefs were previously defined in Table 1. Outliers are represented by symbol (•) and extreme values are represented by symbol (*).

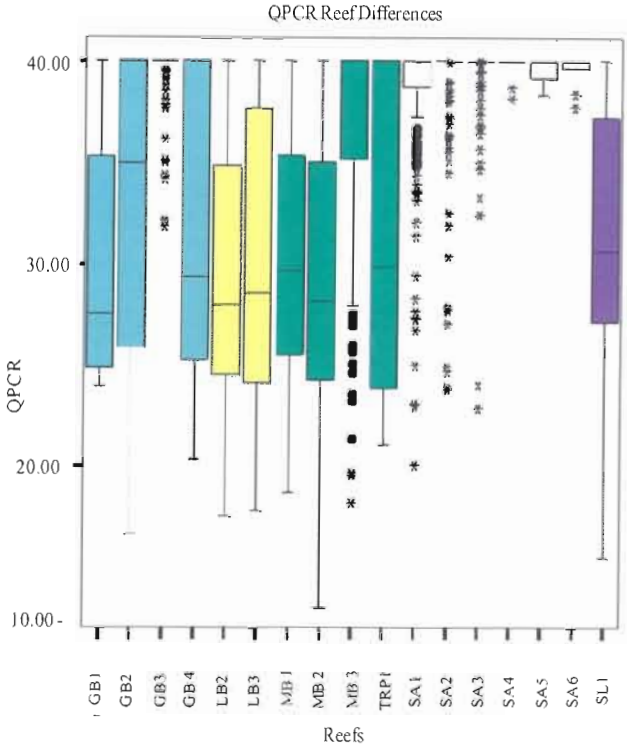

Figure 13. Median, upper, and lower quartiles for QPCR (Ct) values at individual reefs. Reef codes for twelve primary reefs and five supplemental reefs were previously defined in Table 1. Individual bay systems are grouped by color. Outliers are represented by symbol (•) and extreme values are represented by symbol (*).

The box plot median values of San Antonio Bay reefs using RFTM (Figure 12) did not correspond to historic trends of Dermo infection for reefs in this bay system. The box plots for the three San Antonio Bay primary reefs (First Chain Island (SA1), Second Chain Island (SA2), and V-Reef (SA3)), and three supplemental reefs (Panther SA4), Middle Ground (SA5), and Chicken Foot (SA6)) had multiple outliers and extreme values for both methodologies (Figures 12 and 13). Although the number of San Antonio Bay samples that may have been exposed to freezing storage temperatures (< 4° C) cannot be accurately determined, all the tissues from this bay system appeared emaciated or watery in every sample group that was processed every month of the study from October 2009 through November 2010. Although the additional San Antonio Bay samples collected after the study period ended are still being evaluated, the results of San Antonio Bay samples in this study may not be indicative of typical "Dermo responses" to salinity conditions in San Antonio Bay.

Seasonal Differences

The results of QPCR and RFTM were compared for seasonal differences using all data collected during the study period. Seasons were defined as March through May for spring, June through August as summer, September through November as fall, and December through February as winter. Unequal sample sizes were evaluated due to two fall seasons (October - November 2009 and September - November 2010) occurring during the study period (Table 8). In addition two sets of 30 samples were collected and processed for the three San Antonio primary reefs during the second fall season (October 2010) of the study

period. An additional 10 samples were also collected from the Tres Palacios Reef (supplemental reef) and processed with the primary reef samples during the summer season. Inadequate numbers of samples (<10 oysters) were collected at Hanna's Reef and Sammy's Reef during the winter and spring seasons due to excessive mortalities that occurred on both reefs during the study period.

Table 8. Numbers of samples collected each season for evaluating RFTM and QPCR methodologies.

Season	Number of Samples	Percent of Total
Spring	355	21.03
Summer	369	21.86
Fall	612	36.26
Winter	352	20.85
Total Numbers	1,688	100.00

Differences between seasons were evaluated using box plots to show median and upper and lower quartile ranges for seasonal samples using RFTM (Figure 14) and QPCR (Figure 15). The results show that regardless of unequal sample sizes collected each season, median values for both methodologies showed higher levels of Dermo infection during summer and fall seasons (Figures 14 and 15). These results may be an indication of elevated temperature and salinity conditions in each bay system during the summer and fall seasons. Results of QPCR (Figure 15) also show greater infection levels (37 to 32 Ct) and wider distribution of results in all seasons. Wider distribution of Ct values (QPCR) was observed in winter season (Figure 15) than observed for duplicate samples evaluated by RFTM (Figure 14). In addition, lower quartile ranges were 0.0 Mackin scale disease code (RFTM) and 40 Ct (QPCR) were observed for each season indicating oysters not infected by Dermo disease were collected in all samples regardless of seasonal differences.

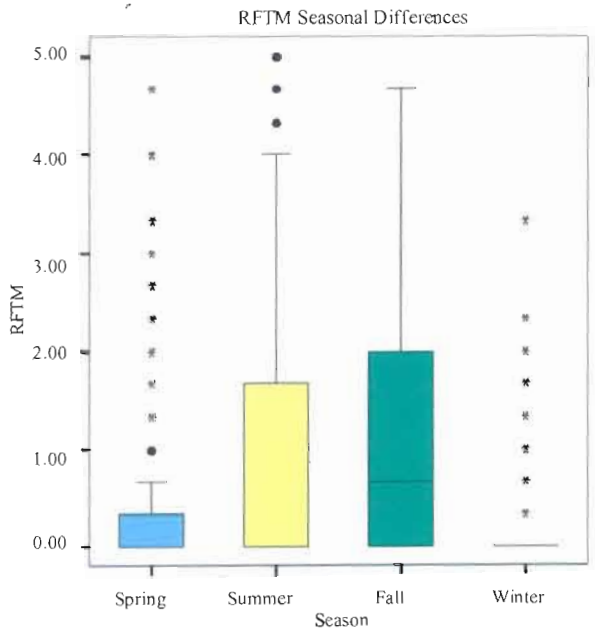

Figure 14. Box plots showing median, upper, and lower quartiles for all RFTM (Mackin scale disease codes) categories during four seasons (spring (March - May), summer (June - August), fall (September - November), winter (December - February)). Outliers are represented by symbol (•), extreme values are represented by symbol (*).

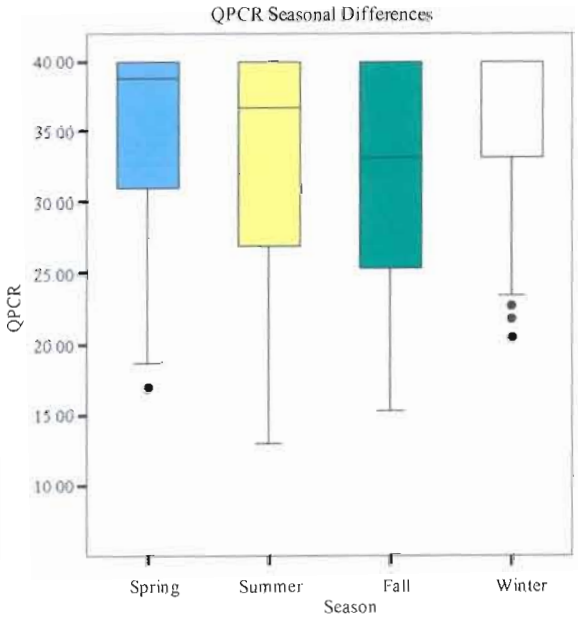

Figure 15. Box plots showing median, upper and lower quartiles for all QPCR (Ct) values during four seasons (spring (March - May), summer (June - August), fall (September - November), winter (December - February)). Outliers are represented by symbol (•), no extreme values were observed.

Individual frequency plots for samples evaluated by RFTM in each season are shown in Figures 16 through 19. These frequency plots show samples evaluated by RFTM are not evenly distributed for each level of infection in all seasons. RFTM detected "no cells" (0.00 Mackin scale disease code) in greater than 250 samples during spring, fall, and winter seasons, whereas it detected less than 200 samples with no infection during the summer season. However RFTM detected significantly higher numbers of "moderate to heavy" infections (2.00 to 5.00 Mackin scale disease codes) in summer and fall seasons when water temperatures were warmer than in spring or winter seasons when water temperatures were lower. The results of this seasonal comparison is consistent with other studies which have documented Dermo infection levels are elevated during higher temperature and salinity conditions in summer and fall seasons; and are lower or suppressed during lower temperatures and salinity conditions in winter and spring seasons (Ray 1987, Burreson and Andrews 1988, Chu and La Peyre 1993, Chu et al. 1993).

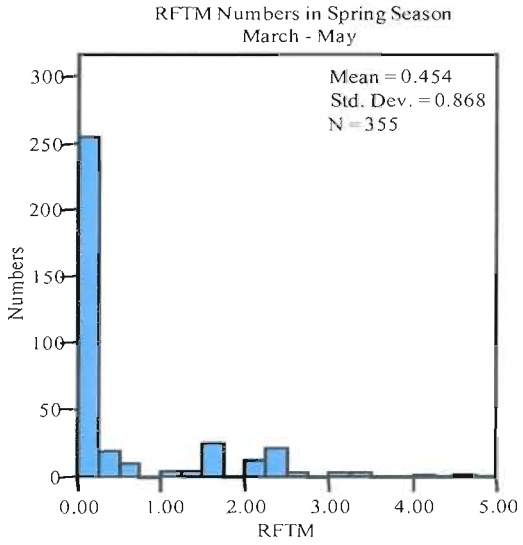

Figure 16. Frequency plot showing number of samples for each RFTM (Mackin scale disease code) category in spring season (March - May).

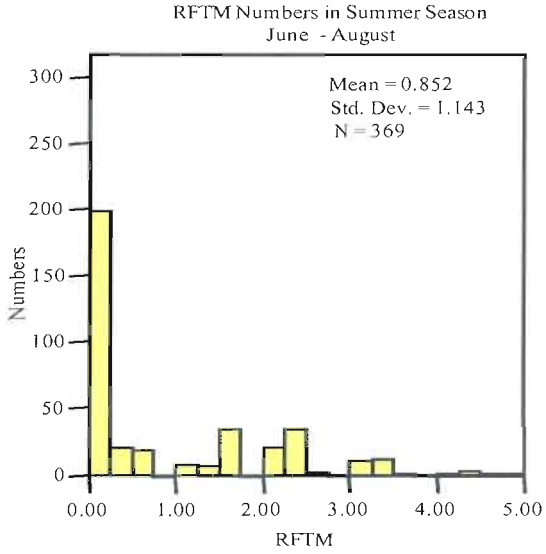

Figure 17. Frequency plot showing numbers of samples for each RFTM (Mackin scale disease code) category in summer season (June - August).

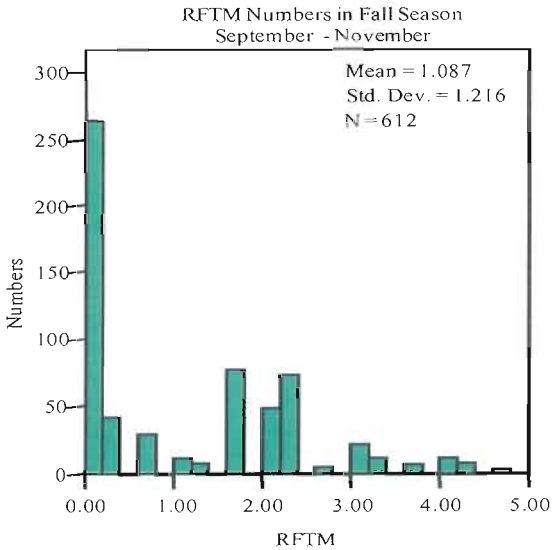

Figure 18. Frequency plot showing number of samples for each RFTM (Mackin scale disease code) category in fall season (September - November).

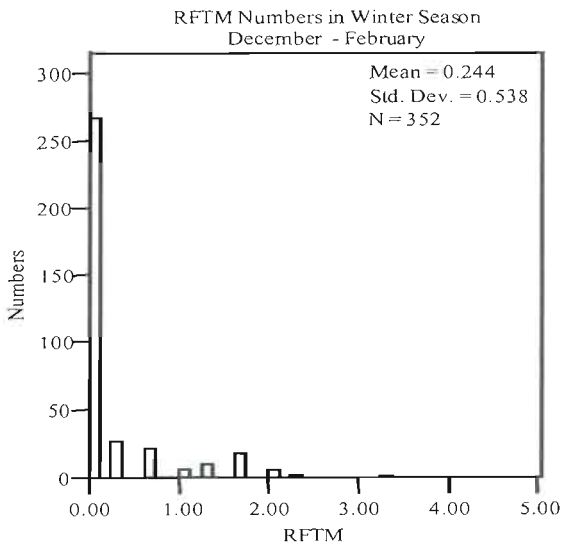

Figure 19. Frequency plot showing numbers of samples for each RFTM (Mackin scale disease code) category in winter season (December - February).

Individual frequency plots for samples evaluated by QPCR in each season are shown in Figures 20 through 23. These frequency plots show QPCR (Ct) samples are evenly distributed for a wide range of infection levels in all seasons. Although more than 200 samples tested negative (40.0 Ct) for Dermo infection using QPCR during winter months, there were greater than 250 samples parallel tested using RFTM that were negative (0.00 Mackin scale disease code) for Dermo infection during these same winter months. Comparison of the two methodologies to detect Dermo infection in the same oyster demonstrates that although RFTM may be limited in detecting very small *P. marinus* cells during the winter months when temperatures are lower and *P. marinus* is naturally suppressed, QPCR was a more sensitive methodology for detecting low level amounts of Dermo infection under the same conditions.

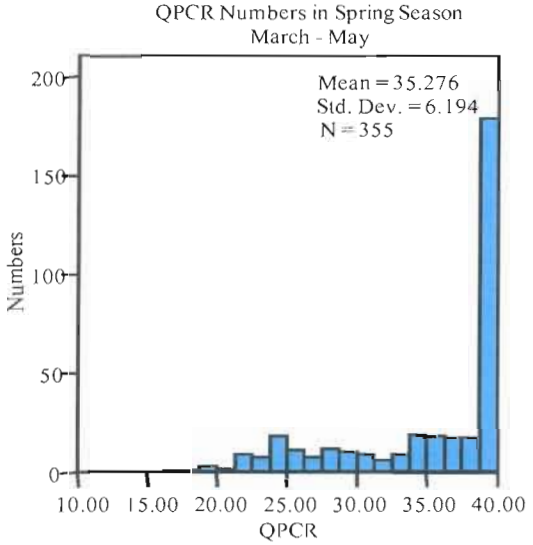

Figure 20. Frequency plot showing number of samples for each QPCR (Ct) value in spring season (March - May).

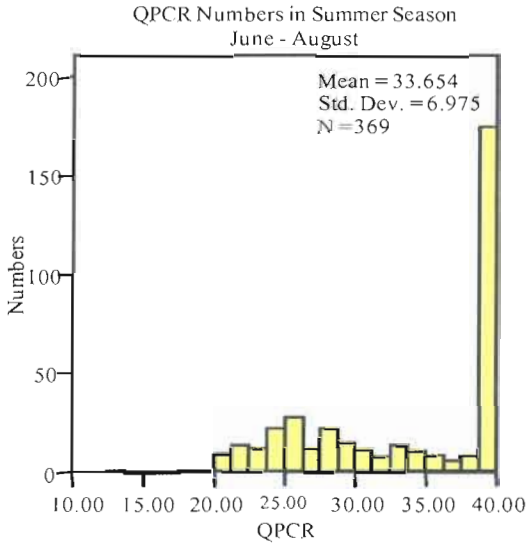

Figure 21. Frequency plot showing number of samples for each QPCR (Ct) value in summer season (June - August).

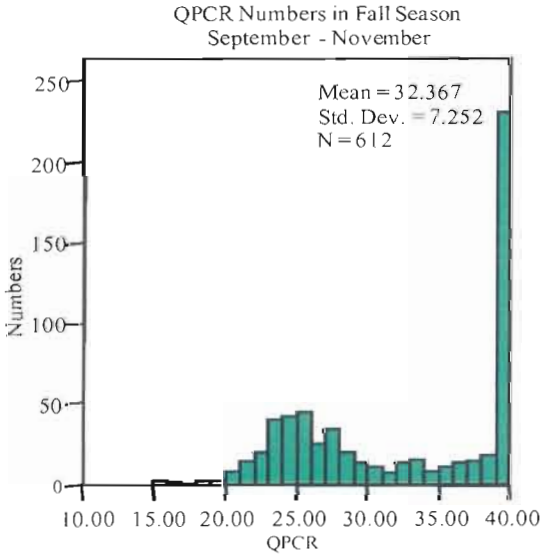

Figure 22. Frequency plot showing number of samples for each QPCR (Ct) value in fall season (September - November).

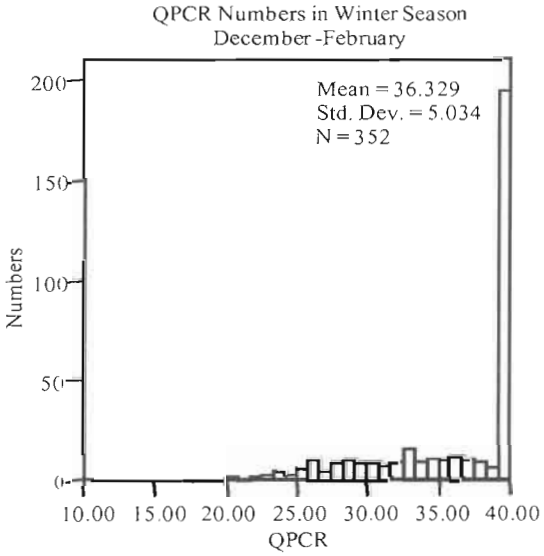

Figure 23. Frequency plot showing number of samples for each QPCR (Ct) value in winter season (December - February).

Salinity Interactions

A scatter plot of Dermo infection levels as measured by RFTM (Mackin scale disease codes) for each sample and the salinity value recorded in the field at the time the sample was collected is shown in Figure 24. These results show fewer oyster samples were collected during the highest salinities (30-35 ppt), whereas the majority of samples were collected during moderate salinities or oyster's physiological optimum salinity range (15-25 ppt). Both high and low levels of Dermo infection as measured by RFTM (Mackin scale disease codes) were observed at these same optimum salinity ranges (15-25 ppt). In addition the number of oysters with the highest Dermo infection levels detected by RFTM (4.65 Mackin scale disease code) were less numerous than samples with lower infection levels (< 2.00 Mackin scale disease codes). These results indicate there may be less "intense" infection in all the populations sampled during this study, or there may be fewer oysters surviving at the highest lethal level of Dermo infection when highest salinity conditions occurred, and therefore less likely to be randomly collected by the dredge samples on each reef. Although not included in this report, historical trends for Dermo infection measured by RFTM show that the numbers of oysters with higher infection levels (>2.00 Mackin scale disease code) increase during long term continuous records of high salinity conditions (TPWD unpublished data). This long term relationship of elevated Dermo infection with long term salinity conditions has been reported by other studies (Ray 1987, Buzan et al. 2009).

The results plotted in Figure 24 also illustrate Dermo infection levels as measured by RFTM (Mackin scale disease codes) appear to increase as salinity increases from 10 to 25 ppt. The division rate of *P. marinus* cells within oysters has been related to both temperature and salinity of the surrounding water conditions (Hofmann et al. 1995). *P. marinus* cell division generally increases with increasing salinities (> 10 ppt), and also increases with increasing temperatures (> 15° C). However *P. marinus* cell growth decreases sharply with salinities < 10 ppt and temperatures < 10° C. Although these general conditions for *P. marinus* cell division rate have been determined under laboratory culture conditions, there are other complex interactions within oyster populations and their environment that may deter or enhance *P. marinus* cell division rate (i.e. density of oyster population on the reef, food availability, gonadal development, recent spawning activity and depletion of tissue weight, etc.). Although higher salinities may affect oyster growth and reproduction, higher oyster mortality rates may be related to both increased Dermo infections (Ray 1987) and increased predation pressure from marine organisms and parasites (Shumway 1996).

A scatter plot of Dermo infection levels as measured by QPCR (Ct) for each sample and the salinity value recorded in the field at the time the sample was collected is shown in Figure 25. These results show similar relationships as those previously described for Dermo infection measured by RFTM. Fewer samples were collected during the highest salinities (30-35 ppt), whereas the majority of samples were collected during moderate salinities or oyster's physiological optimum salinity range (15-25 ppt). Lower QPCR (12-15 Ct) values indicating high Dermo infection levels have a similar relationship as Mackin scale disease codes where infection increases as salinity increases from 10 to 25 ppt. Low Dermo infection levels indicated by high QPCR (30-40 Ct) values also appeared to be related to decreased salinities (0-10 ppt). These results also indicate there may be less "intense" infection in all the populations sampled, or there may be fewer oysters surviving at the highest lethal level of Dermo infection when highest salinity conditions occurred, and therefore less likely to be randomly collected by the dredge samples on each reef.

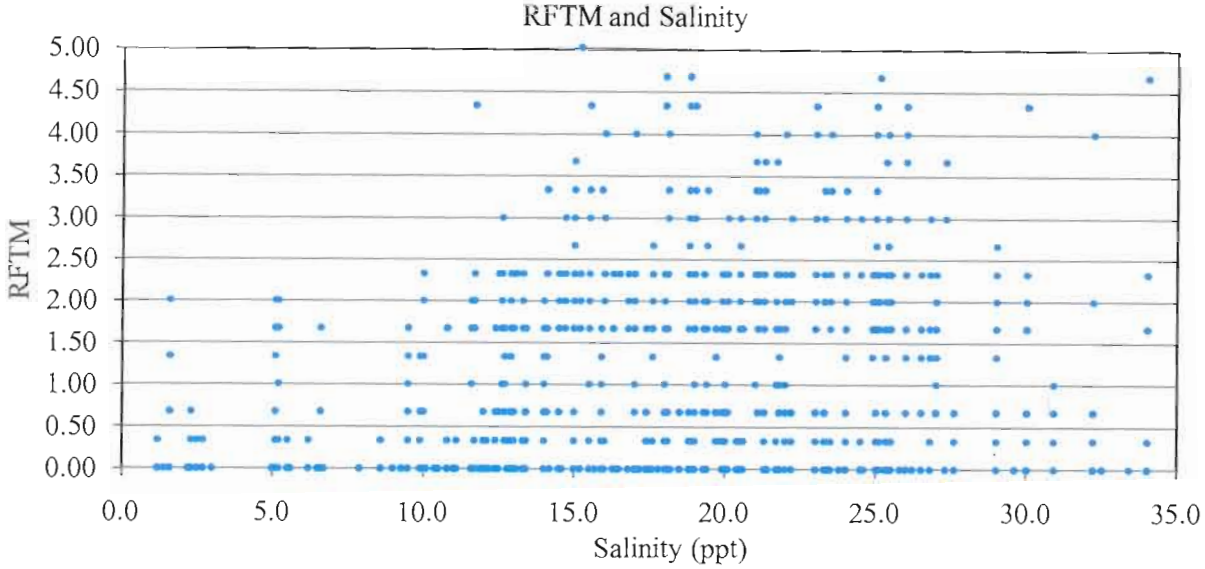

Figure 24. Scatter plot of Dermo infection (Mackin scale disease code) as measured by RFTM at different salinities (ppt).

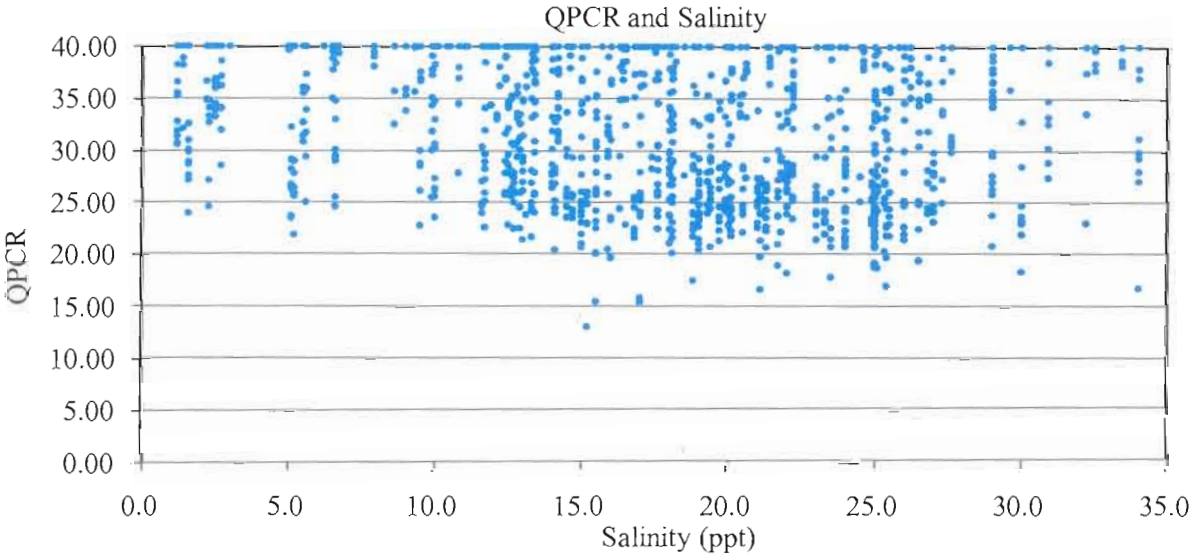

Figure 25. Scatter plot of Dermo infection (Ct) as measured by QPCR at different salinities (ppt). High Dermo infection is measured by low Ct values. Low Dermo infection is measured by high Ct values.

Temperature Interactions

A scatter plot of Dermo infection levels as measured by RFTM (Mackin scale disease codes) for each sample and the temperature recorded in the field at the time the sample was collected is shown in Figure 26. Although there are a wide range of RFTM (Mackin scale disease codes) for moderate to high temperatures (15-30° C), Dermo infection levels appear to increase as temperatures increase (20 to 35° C) (Figure 26). Dermo infection levels as measured by RFTM (Mackin scale disease codes) also appear to be reduced at lower temperatures (<10° C).

A scatter plot of Dermo infection levels as measured by QPCR (Ct) for each sample and the temperature recorded in the field at the time the sample was collected is shown in Figure 27. Although there are a wide

range of QPCR (Ct) values for moderate to high temperatures (15-30° C), high Dermo infection levels (12-20 Ct) appear to increase as temperatures increase (20-35° C) (Figure 27). Although QPCR (Ct) results associated with moderate to high temperatures are comparable with Dermo infection levels as measured by RFTM (Mackin scale disease codes), there are subtle differences observed for QPCR's ability to detect low levels of Dermo infection at lower temperature ranges (<10° C). These results confirm QPCR has greater sensitivity to detect Dermo infection in colder water temperatures.

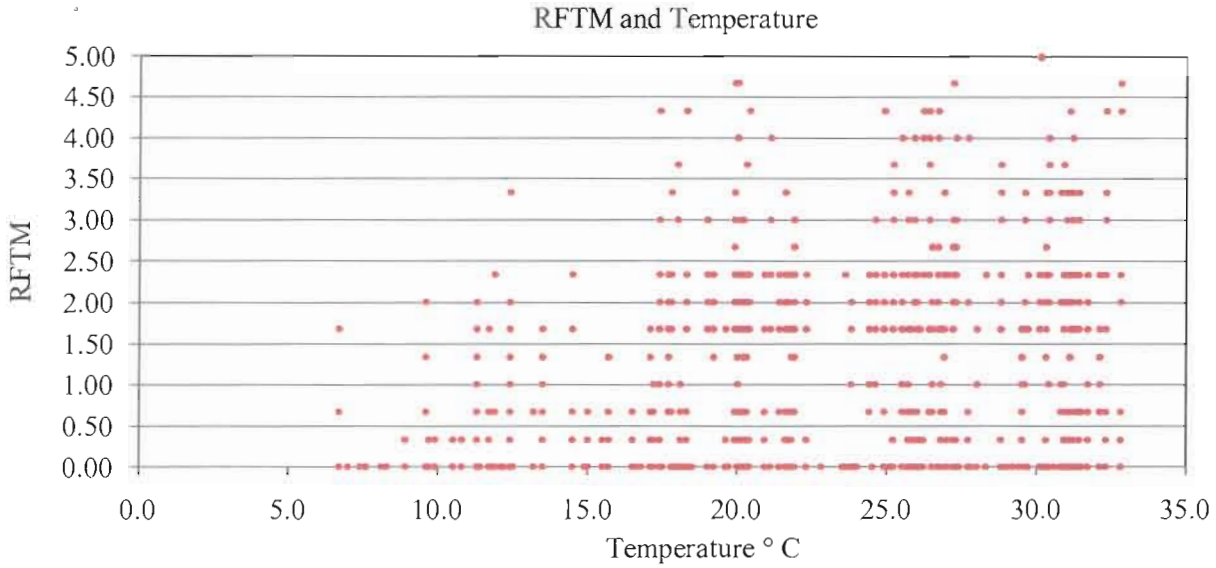

Figure 26. Scatter plot of Dermo infection (Mackin scale disease code) as measured by RFTM at different temperatures (°C).

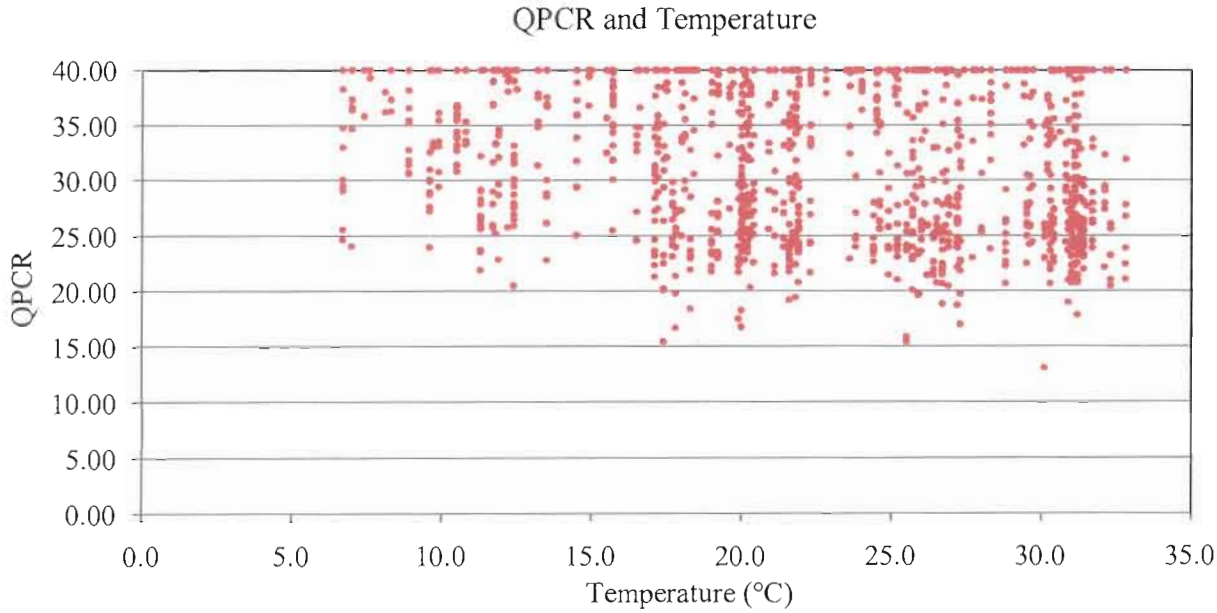

Figure 27. Scatter plot of Dermo infection (Ct) as measured by QPCR at different temperatures (°C). High Dermo infection is measured by low Ct values. Low Dermo infection is measured by high Ct values.

DISCUSSION

The ability to detect disease and quantify infection intensity in oysters is not a new approach. Diagnostic methods have been available since the 1950's (Ray 1952) and various real-time assays have previously been described (Yarnall et al. 2000, Audemard et al. 2004, Gauthier et al. 2006). However, laboratory methodologies typically require modification and optimization in order to account for institutional capability as well as heterogeneity of laboratory conditions. The goal of the current study was to compare QPCR (Ct) and RFTM (Mackin scale disease codes) results from oyster populations in five bay systems over a thirteen month study period. An additional goal was to standardize these results in order to preserve the continuity between historical and future data sets of Dermo infection in Texas oyster populations.

The evaluation of all 1,688 samples from five bay systems provided a clear linear inverse relationship that implies QPCR (Ct) values are comparable for a wide range of RFTM (Mackin scale disease codes) categories for all samples as shown in Figure 3. Average QPCR (Ct) results correlate better with traditional RFTM (Mackin scale disease codes) results using linear regression methods. However, comparison of the two methodologies to detect Dermo infection in the same oyster or from different bay systems in this study show specific transport and storage protocols need to be maintained for standardization of tissue sample condition when comparing RFTM and QPCR results. Additionally, comparison of the two methodologies to detect Dermo infection in the same oyster demonstrates that although RFTM may be limited in detecting very small *P. marinus* cells during the winter months when temperatures are lower, QPCR was a more sensitive methodology for detecting low level amounts of Dermo infection under the same conditions.

The main advantage of real-time molecular methods over traditional culture (RFTM) methods is that QPCR is more sensitive to detecting Dermo infections at all water temperatures and under all sample storage conditions. It is also not dependent on the scientific expertise of the investigator reading individual slides. QPCR has the advantage that samples can be stored for longer periods of time (greater than one year if refrigerated). RFTM requires the slides are read within a 30-60 days following the seven day incubation period.

Although this study extracted and processed 120 oyster samples per month, QPCR has the advantage of being able to isolate and determine Dermo infection levels in large groups of samples (96 samples per QPCR plate). This methodology reduces the time and effort required by one person to obtain a comparable result with RFTM requirements to determine infection levels. The entire process of extracting, isolating, and quantifying the *P. marinus* DNA from 96 mantle tissues may be possible over a three day period after collection of samples. Currently mantle tissues could be extracted using sterilized equipment anywhere the oyster samples are collected, and stored in 95% alcohol for extended periods of time until there are sufficient numbers of samples (96) to run using QPCR procedures. The QPCR batch processing and quantification of samples contrasts with the traditional RFTM culture procedure, which includes time-consuming individual visual inspection of each slide by a qualified expert in Dermo cell diagnostics after each sample has been incubated over a seven day period of time. The QPCR methodology also removes the subjectivity of the RFTM results which are generally based on semi-quantitative assessment, and are subject to human error.

One potential disadvantage of real-time QPCR methodologies for assessing parasite presence and intensity is that real time threshold cycle time (Ct) is not directly related to parasite load. Thus QPCR results must be calibrated using either *P. marinus* cell standards or with duplicate samples that are processed using RFTM for each processed sample. However, the biggest advantage of QPCR is its sensitivity to the target DNA. Whereas the traditional culture technique (RFTM) relies on semi-quantitative categorical classification of direct counts of the parasite cells (at low levels) or estimation of parasite cell counts (at high levels), the molecular technique of QPCR quantitatively "measures" the amount of target DNA present. This methodology measures both the DNA that is a live functional parasite and one that has degraded. If the

target DNA is present, then QPCR recognizes that DNA template. Consequently QPCR (Ct) values need to be standardized with the same samples with a "known parasitic burden" using RFTM. This standardization procedure is important for two interrelated reasons. First, the Mackin scale disease code, based on parasitic cell counts, can be predictive of disease morbidity and epizootiology. Thus, QPCR (Ct) results compared to standardized samples representing a range of Mackin scale disease codes have a more predictive or management utility (as previously demonstrated in Figure 3). Second, standardized samples are necessary to quantify any variability of the QPCR assay between runs. This standardization step is important so that 1) samples from one date can be directly compared to samples from another assay at an earlier or later date, and 2) reagent or cycling problems with the assay can be identified by comparison of standard samples to identical samples from previous runs.

Standardization Challenges

Sampling errors may occur when attempting to extract the same volume of tissue from each oyster. The study results show there were issues with standardizing the volume or mass of tissues extracted from the anterior mantle area that would be representative of samples for testing both methodologies. Various scenarios were tested during this study year by taking the same side of the anterior mantle tissue, switching sides of the anterior mantle for each methodology, and macerating larger volumes of samples for QPCR prior to sub-sampling for isolation procedures. Although the mantle veins were not always visible, the densities of veins on the right and left sides of "transparent" watery tissues were documented during the tissue extraction procedure. Initial analysis of this data does not show any significant differences between samples with or without high densities of veins. However, the standardization of tissue size (mass and volume) evaluated by each methodology has not been resolved so that results can be replicated and runs are more consistent and comparable to one another.

Although processing samples for Dermo in the whole body burden of the tissues would account for the differences in sizes of the tissues in each oyster, this method may be a very time consuming process. In the case of Sabine Lake oysters, the quality of the tissues examined in this study were often degraded (discolored and constricted) and the actual amount of Dermo infection in duplicate samples may not have been accurately derived. Additional evaluation of San Antonio Bay oysters will also need to be done before applying a linear response equation to compare Dermo infection using QPCR and RFTM methodologies.

The effect of seasonal temperature changes on the results of RFTM and QPCR evaluation methodologies must also be a consideration for future studies. Seasonal differences evaluated in this study indicated colder winter temperatures (<10° C) suppressed *P. marinus* cells from propagating and hence there were generally lower levels of infection in all oyster populations. However the results of the QPCR showed the molecular method had higher sensitivity to detecting *P. marinus* cells and was able distinguish low levels of Dermo infection, whereas RFTM was not able to detect Dermo infection in oysters from low water temperatures (<10° C) or from oyster samples that have been previously frozen. The capacity of QPCR to detect Dermo infection in oysters that were chilled to below optimum environmental levels (including frozen samples) in this study provides an important logistical consideration for future sample storage protocols. Although RFTM appears to require important care and management of the samples collected in order to accurately detect Dermo infection levels, QPCR does not require the same amount of sample storage control measures to be able to detect Dermo infection levels. Sample storage issues should be considered to devise an economically feasible storage protocol for samples collected from widely dispersed locations that are far from the location from where they are to be processed.

Alternative Standardization

There are two other possible methodologies for further development of diagnostic standards for determining *P. marinus* using QPCR. In each case, standard samples must be included in the same sample plate as experimental samples, for each QPCR assay. The two methodologies differ mainly in how standard

samples are produced and/or maintained. First, standards can be made by acquiring oysters of different infection levels that have been diagnosed using the standard RFTM culture methodology. This would require long-term maintenance of oyster tissues at optimum conditions. When standard DNA samples run out, new DNA would need to be isolated and quantified from the same oysters. One major problem with this strategy is that the culture methodology could never be fully abandoned, as each oyster standard would need to be quantified using traditional techniques. A second setback is that standard sample accuracy would be completely reliant upon accurate use of RFTM and semi-quantitative diagnosis of the infection levels. Finally, maintenance of tissue samples over long periods, coupled with multiple freeze/thaw events, could eventually lead to tissue degradation, and unexpected variability in standard runs.

A second methodology for standardization would be to prepare artificial DNA standards by inoculating oyster tissue with a known volume of *P. marinus* cells, as described by Gauthier et al. (2006). Briefly, this methodology involves culturing *P. marinus* in the lab, and estimating the density of cells in culture using a hemocytometer. Uninfected oyster tissue would be inoculated with a predetermined number of *P. marinus* cells, and the DNA isolation procedure would be immediately carried out on the tissue/cell mix. Different volumes of *P. marinus* cells can be used to simulate a range of infection intensity in any number of standards (parasites/g wet weight of oyster). The advantage of this methodology is that standards can be regenerated indefinitely without need for microscope examination of samples. The main disadvantage of this methodology is that it requires live cultures of parasite cells whenever standards are prepared. Although cultured *P. marinus* cells are available commercially, long-term studies would require either that cells be ordered on numerous occasions or that cultures be maintained in the lab.

MANAGEMENT IMPLICATIONS

The assessment of *P. marinus* prevalence and intensity in natural oyster populations using real-time QPCR methodology is a promising tool for evaluation of ecosystem health. As previously stated, the persistence of *P. marinus* is strongly affected by estuarine water temperature and salinity; thus ecosystem effects of reduced inflow and climatic events can be accessed through monitoring of parasite prevalence over time. The QPCR methodology of Gauthier et al. (2006) is appropriate for such a goal.

The variability between individual results and between individual bay system results indicate specific transport and storage protocols need to be maintained for standardization of tissue sample condition when comparing RFTM and QPCR results. Comparison of the two methodologies to detect Dermo infection in the same oyster demonstrates that although RFTM may be limited in detecting very small *P. marinus* cells during the winter months when temperatures are lower, QPCR is a more sensitive methodology for detecting low level amounts of Dermo infection under the same conditions.

In addition, a methodology must be developed whereby QPCR (Ct) values are translated into a biological context in order for molecular methodologies to be applicable to management of the resource. In this study that complex issue has not been adequately explored and will require additional evaluation of the data we have collected and have not yet analyzed since the study funded by TWDB ended.

In conclusion, the duplicate samples that have been assayed using RFTM and rated using Mackin scale of disease codes are comparable with QPCR (Ct) results. These results have also been successfully used to determine direct or inverse relationships with salinity and temperatures, reef's proximity to freshwater sources, and bay systems.

FUTURE DIRECTIONS: *Oysters as Sentinels of Freshwater Inflows*

In conclusion, TPWD considers QPCR an appropriate methodology for monitoring intensity and presence of Dermo infection in oyster populations along the Texas coast. Currently TPWD is evaluating which reefs would be the most appropriate target reefs for monitoring Dermo disease that provide both temporal and

spatial components related to freshwater sources or other management strategies. TPWD is also examining the potential for reducing the number of samples collected from each bay system to a more manageable and economically viable size than is currently being done. One option that is being considered is targeting one representative oyster population for each bay system as a sentinel reef. Although monthly monitoring of these sentinel reefs is preferred when comparing biological variables to continuous freshwater inflow time series data, there are economic constraints on state agencies that would require reducing monitoring to quarterly sampling efforts. In addition, the use of only QPCR to monitor Dermo infection may require at least annual validation by RFTM. Although more costly and time consuming, there also needs to be further assessment of using whole body burden assays (La Peyre et al. 2009) to eliminate the potential for false negatives when Dermo infection may in fact be present in other body tissues than the anterior mantle tissue. TPWD will be evaluating the current study's data in addition to the data collected since the study period ended to formalize any future monitoring program's protocols and interpretation of results. TPWD will also be evaluating the value of continuing to collect samples for monitoring Dermo infection using QPCR in the future.

This study also identified several challenges to be considered in future Dermo monitoring programs:

- Standardization of tissue volume being evaluated.
- QPCR appears to have greater sensitivity to detect Dermo infections whereas RFTM appears to have less sensitivity (bay temperatures (<10°C) suppresses *P. marinus* cell division).
- Mantle tissue extraction is difficult in emaciated oysters or oysters with excessive gonadal tissues.
- Mantle veins are not equally distributed or always in same location within the anterior end of the mantle resulting in slightly different amounts of Dermo infection in duplicate samples.
- Cost efficiency for QPCR is reduced when fewer samples are run than the maximum number (96) of samples per plate.
- Potential interferences from gonadal development or glycogen in tissues may interfere with collection of tissue or DNA isolation procedures.

This study also provided beneficial information that could improve the biological/ecological monitoring that TPWD currently does through their resource monitoring program. In this study, the oyster's internal tissue (meat) condition as well as its external shell (bill) condition were examined and recorded. This information could be used to determine environmental or parasite related influences on the oyster's health. Watery or emaciated oyster tissues appeared to be indications of negative environmental or parasite related stress. Fatty tissues or excessive gonadal tissues appeared to be positive indications of optimum environmental conditions or less parasite related influences on the population. Sharp or overlapping shells without holes from *Polydora* (polychaete) mud worms, boring clams or sponges were considered indications of recent growth of the oyster without environmental or parasite related detriments. Blunt or retro-regressed shells or shells full of holes from *Polydora* mud worms, or from boring clams or sponges were indications the oysters had been subjected to saltier environmental conditions or excessive parasites. In addition during spring, summer and fall seasons the sex of the oyster could be determined through examination of the gonad material. The male to female ratio in market-sized oysters is an important indicator of stress from environmental conditions. Oysters are protandric species and change from male to females as they grow from juvenile to market size class. Therefore more males are normally present in the juvenile size class and more females are present in the market size class. More males are also found in the market size class when environmental conditions are not optimum and females have reverted back to males as a survival mechanism (Kennedy et al. 1996).

During this study additional categorical information was recorded on the abundance of attached benthic organisms associated with oysters (hooked mussels, Florida rocksnail, boring clams, boring sponges, slipper shells, tunicates, *Polydora* mud worms, oyster drills, etc.). Although this information is not included in this report, TPWD will be evaluating the benefits of using the abundance or absence of these organisms as

potential indicator organism for changing salinity conditions that are not normally recorded in the TPWD database or evaluated through the resource monitoring program.

In conclusion, this study has provided TPWD a viable alternative for monitoring Dermo infection in Texas Bay systems through the use of QPCR standardized by RFTM. TPWD considers QPCR an appropriate monitoring methodology for measuring intensity and presence of Dermo infection in oyster populations along the Texas coast.

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Appendix A. Dermo DNA Isolation Procedures

Oyster DNA isolation procedure for real-time PCR diagnosis of *Perkinsus marinus*, using Purgene® mini-prep kits.

1. Remove oyster tissues from 10-ml storage container and place on clean paper towels using sterilized forceps.
2. Partially dry tissues and macerate and cut up tissues with sterilized forceps and scissors until well mixed.
3. Subsample the mixed partially dry tissues and place inside a 1.5 ml sterilized tube.
4. Spot 5 µl of proteinase K (20 mg/ml) on the inside wall of a 1.5 ml tube.
5. Wash the proteinase K down into the tube with 600 µl of cell lysis buffer (provided in kit).
6. Cut off 50 mg of oyster mantle tissue, placing it into the 1.5 ml tube.
7. Using a fine-point dissection pin, mince the tissue inside the tube until it has broken into multiple smaller fragments.
8. Incubate the sample overnight at 55° C, with occasional vortex of samples.
9. Check samples to see that they have dissolved completely, leaving a semi-clear liquid in the tube. For samples which are cloudy, add 10 µl more of proteinase K and continue incubation until clear.
10. Place samples in an ice bath and cool. Once they have cooled, add 200 µl of protein precipitation solution (provided in kit), and return to ice bath for 15-30 minutes.
11. To a second labeled tube, add 700 µl of undiluted isopropanol.
12. Centrifuge sample from step # 8 at max speed (=14000 rpm) for 3 minutes.
13. Pour the supernatant into the 2nd tube containing isopropanol.
14. Invert sample multiple times to mix, then put sample in freezer for 1 hour.
15. Spin sample at max speed for 3 minutes.
16. Pour off supernatant, then add 600 µl of 70% ethanol to wash DNA pellet. Wash pellet by inverting sample multiple times.
17. Spin sample at max speed for 1 minute.
18. Pour off supernatant, then dry sample upside down on bench top on a piece of absorbent paper for 15-30 minutes. Examine tubes for any latent moisture before adding rehydration solution.
19. Rehydrate samples in 100 to 250 µl of DNA rehydration solution (commercial). Rehydrate overnight and transport to PRBMRL.
20. Samples can be stored refrigerated for multiple days prior to processing for QPCR evaluation.

Appendix B. Statistical Results

Table B1. Descriptive statistics for RFTM (Mackin scale disease codes) and QPCR (Ct) in all samples (n=1,688).

Methodology	Mean	Std. Deviation
QPCR	34.087	6.740
RFTM	0.727	1.074

Table B2. Coefficient of determination for RFTM (Mackin scale disease codes) and QPCR (Ct) in all samples (n=1,688).

Model	r	r^2	Adjusted r^2	Std. Error	Durbin-Watson
1	0.862(a)	0.743	0.743	3.419	1.323

Table B3. Analysis of variance of RFTM (Mackin scale disease codes) and QPCR (Ct) in all samples (n=1,688).

Model	Statistic	Sum of Squares	df	Mean Square	F	Sig.
1	Regression	56,915.381	1	56,915.381	4,867.689	0.000
	Residual	19,713.529	1686	11.692		
	Total	76,628.909	1687			

Table B4. Summary of coefficients for dependent variable QPCR (Ct) in all samples (n=1,688).

Statistic	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Intervals	
Model	B	Std. Error	Beta			Lower Bound	Upper Bound
1 (Constant)	38.018	0.101		378.263	0.000	37.821	38.215
RFTM RF	-5.407	0.078	-0.862	-69.769	0.000	-5.559	-5.255

Table B5. Pearson correlation coefficient for RFTM (Mackin scale disease codes) and QPCR (Ct) in all samples (n=1,688).

Methodology	Pearson Correlation	Significance (1-tailed)
QPCR	1.000	0.000
RFTM	-0.862	0.000

Table B6. Residuals statistics of dependent variable QPCR (Ct) in all samples (n=1,688).

Statistic	Minimum	Maximum	Mean	Std. Deviation
Predicted Value	10.981	38.018	34.087	5.808
Std. Predicted Value	-3.978	0.677	0.000	1.000
Standard Error of Predicted Value	0.083	0.341	0.112	0.035
Adjusted Predicted Value	10.961	38.031	34.086	5.811
Residual	-15.248	14.731	0.000	3.418
Std. Residual	-4.459	4.308	0.000	1.000
Student Residual	-4.461	4.321	0.000	1.000
Deleted Residual	-15.261	14.822	0.001	3.423
Student Deleted Residual	-4.486	4.344	0.000	1.002
Mahal's Distance	0.003	15.824	0.999	1.708
Cook's Distance	0.000	0.057	0.001	0.003
Centered Leverage Value	0.000	0.009	0.001	0.001

Table B7. Summary of coefficients for dependent variable QPCR (Ct) in Sabine Lake samples (n=140).

Statistic	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Intervals	
Model	B	Std. Error	Beta			Lower Bound	Upper Bound
1 (Constant)	35.346	0.464		75.156	0.000	34.428	36.263
RFTM	-3.779	0.298	-0.734	-12.695	0.000	-4.367	-3.190

Table B8. Analysis of variance of RFTM (Mackin scale disease codes) and QPCR (Ct) in Sabine Lake samples (n=140).

Model	Statistic	Sum of Squares	df	Mean Square	F	Sig.
1	Regression	2,775.911	1	2,775.912	161.173	0.000
	Residual	2,376.805	138	17.223		
	Total	5,152.716	139			

Table B9. Summary coefficients for dependent variable QPCR (Ct) in Galveston Bay Samples (n=389).

Statistic	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Intervals	
Model	B	Std. Error	Beta			Lower Bound	Upper Bound
1 (Constant)	38.494	0.198		194.573	0.000	38.105	38.883
RFTM	-5.642	0.162	-0.871	-34.845	0.000	-5.959	-5.323

Table B10. Analysis of variance for RFTM (Mackin scale disease codes) and QPCR (Ct) for Galveston Bay samples (n=389).

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	12,940.548	1	12,940.548	1,214.170	0.000
	Residual	4,124.626	387	10.658		
	Total	17,065.174	388			

Table B11. Summary coefficients for dependent variable QPCR (Ct) in Matagorda Bay samples (n=429).

Statistic	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Intervals	
Model	B	Std. Error	Beta			Lower Bound	Upper Bound
1 (Constant)	37.070	0.215		172.009	0.000	36.647	37.494
RFTM	-4.976	0.139	-0.866	-35.891	0.000	-5.249	-4.704

Table B12. Analysis of variance of RFTM (Mackin scale disease codes) and QPCR (Ct) for Matagorda Bay samples (n=429).

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	15,253.612	1	15,253.612	1,288.180	0.000
	Residual	5,056.200	427	11.841		
	Total	20,309.812	428			

Table B13. Summary coefficients of dependent variable QPCR (Ct) for Lavaca Bay samples (n=280).

Statistic	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Intervals	
Model	B	Std. Error	Beta			Lower Bound	Upper Bound
1 (Constant)	36.077	0.348		103.629	0.000	35.391	36.762
RFTM	-4.867	0.209	-0.813	-23.269	0.000	-5.279	-4.455

Table B14. Analysis of variance of RFTM (Mackin scale disease codes) and QPCR (Ct) for Lavaca Bay samples (n=280).

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	7,829.693	1	15,253.612	541.437	0.000
	Residual	4,020.147	278	11.841		
	Total	11,849.840	279			

Table B15. Summary coefficients of dependent variable QPCR (Ct) for San Antonio Bay samples (n=450).

Statistic	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Intervals	
Model	B	Std. Error	Beta			Lower Bound	Upper Bound
1 (Constant)	39.0337	0.098		400.089	0.000	39.143	39.530
RFTM	-5.405	0.202	-0.784	-26.735	0.000	-5.802	-5.008

Table B16. Analysis of variance of RFTM (Mackin scale disease codes) and QPCR (Ct) for San Antonio Bay samples (n=450).

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	2,968.092	1	2,968.090	714.769	0.000
	Residual	1,860.329	448	4.153		
	Total	4,828.421	449			

Table B17. Descriptive statistics for Dermo infection by RFTM method for each reef.

Statistic	Sabine	Hanna's	Fisher's	April Fool's	Sammy's	Mad Island	Shell Island
Mean	1.021	0.974	0.000	1.126	1.269	1.307	0.352
Std. Error	0.100	0.109	0.000	0.093	0.095	0.106	0.076
95% Lower CI	0.824	0.759	0.000	0.942	1.082	1.097	0.202
95% Upper CI	1.219	1.190	0.000	1.309	1.456	1.517	0.502
Median	0.330	0.330	0.000	1.000	1.330	1.330	0.000
Variance	1.399	1.389	0.000	1.116	1.251	1.574	0.800
Std. Deviation	1.183	1.178	0.000	1.056	1.119	1.255	0.894
Minimum	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Maximum	4.670	4.330	0.000	4.330	4.670	5.000	4.330
Range	4.670	4.330	0.000	4.330	4.670	5.000	4.330
Interquartile Range	2.000	1.840	0.000	2.000	2.330	2.330	0.000
Skewness	0.907	0.878	0.000	0.546	0.502	0.651	2.952
Kurtosis	-0.074	-0.362	0.000	-0.591	-0.419	-0.373	8.337

Table B17. (continued) Descriptive statistics for Dermo infection by RFTM method for each reef.

Statistic	Tres Palacios	Gallinipper	Indian Point	First Chain Isl.	Second Chain Isl.	V-Reef
Mean	1.466	1.2479	1.2736	0.207	0.082	0.022
Std. Error	0.569	0.0850	0.0987	0.058	0.029	0.016
95% Lower CI	0.178	1.0798	1.0784	0.092	0.025	-0.009
95% Upper CI	2.754	1.4161	1.4689	0.321	0.139	0.054
Median	0.500	1.6700	1.6700	0.000	0.000	0.000
Variance	3.240	1.0120	1.3650	0.501	0.125	0.038
Std. Deviation	1.800	1.0062	1.1683	0.708	0.354	0.195
Minimum	0.000	0.0000	0.0000	0.000	0.000	0.000
Maximum	4.670	4.6700	4.3300	4.000	2.330	2.330
Range	4.670	4.6700	4.3300	4.000	2.330	2.330
Interquartile Range	2.830	1.6700	2.3300	0.000	0.000	0.000
Skewness	1.055	0.3510	0.4680	3.851	4.814	11.306
Kurtosis	-0.371	-0.4150	-0.7210	14.460	23.194	133.369

Table B18. Descriptive statistics for Dermo infection by QPCR for each reef.

Statistic	Sabine	Hanna's	Fisher's	April Fool's	Sammy's	Mad Island	Shell Island
Mean	31.487	32.841	39.596	31.080	30.555	29.482	36.499
Std. Error	0.515	0.642	0.114	0.583	0.507	0.576	0.474
95% Lower	30.470	31.569	39.371	29.926	29.554	28.344	35.562
95% Upper	32.504	34.112	39.821	32.233	31.557	30.621	37.435
Median	30.640	34.440	40.000	29.320	29.660	28.185	40.000
Variance	37.070	48.226	1.843	44.182	35.918	46.419	31.170
Std. Deviation	6.089	6.944	1.358	6.647	5.993	6.813	5.583
Minimum	15.380	16.600	31.840	20.390	18.660	12.980	18.200
Maximum	40.000	40.000	40.000	40.000	40.000	40.000	40.000
Range	24.620	23.400	8.160	19.610	21.340	27.020	21.800
Interquartile Range	10.130	14.290	0.000	14.760	9.830	10.720	4.940
Skewness	-0.081	-0.355	-4.097	0.179	0.242	0.136	-1.613
Kurtosis	-0.819	-1.409	17.335	-1.470	-1.183	-0.906	1.547

Table B18. (continued) Descriptive statistics for Dermo infection by QPCR for each reef.

Statistic	Tres Palacios	Gallinipper	Indian Point	First Chain Isl.	Second Chain Isl.	V-Reef
Mean	31.372	29.5926	30.288	38.271	38.661	39.398
Std. Error	2.531	0.5310	0.570	0.317	0.280	0.182
95% Lower	25.647	28.5428	29.160	37.645	38.109	39.039
95% Upper	37.097	30.6425	31.415	38.897	39.214	39.756
Median	29.850	27.9450	28.505	40.000	40.000	40.000
Variance	64.057	39.4690	45.538	15.066	11.739	4.941
Std. Deviation	8.004	6.2825	6.748	3.881	3.426	2.223
Minimum	21.030	17.4300	17.780	20.070	23.810	22.870
Maximum	40.000	40.0000	40.000	40.000	40.000	40.000
Range	18.970	22.5700	22.220	19.930	16.190	17.130
Interquartile Range	16.520	10.3400	13.630	1.320	0.000	0.000
Skewness	0.046	0.4550	0.274	-2.706	-3.087	-5.592
Kurtosis	-2.005	-1.0440	-1.366	7.155	9.019	35.756

Appendix C. TWDB's Executive Administrator's Draft Report Comments

Texas Water Development Board

P.O. Box 13231, 1700 N. Congress Ave.
Austin, TX 78711-3231, www.twdb.state.tx.us
Phone (512) 463-7847, Fax (512) 475-2053

August 24, 2011

Mr. Scott Boruff
Deputy Executive Director of Operations
Texas Parks and Wildlife Department
4200 Smith School Road
Austin, Texas 78744

Re: Interagency Cooperation Contract between the Texas Water Development Board (TWDB) and the Texas Parks and Wildlife Department (TPWD); TWDB Contract No. 1004831018, Draft Report Comments

Dear Mr. Boruff:

Staff members of the TWDB have completed a review of the draft report prepared under the above-referenced contract. ATTACHMENT I provides the comments resulting from this review. As stated in the TWDB contract, TPWD will consider incorporating draft report comments from the EXECUTIVE ADMINISTRATOR as well as other reviewers into the final report. In addition, TPWD will include a copy of the EXECUTIVE ADMINISTRATOR'S draft report comments in the Final Report.

The TWDB looks forward to receiving one (1) electronic copy of the entire Final Report in Portable Document Format (PDF) and six (6) bound double-sided copies. TPWD shall also submit one (1) electronic copy of any computer programs or models, and, if applicable, an operations manual developed under the terms of this Contract.

If you have any questions concerning the contract, please contact Dr. Carla Guthrie, the TWDB's designated Contract Manager for this project at (512) 463-4179.

Sincerely,

Robert E. Mace, Ph.D., P.G.
Deputy Executive Administrator
Water Science and Conservation

Enclosures

c: Carla Guthrie, Ph.D., TWDB

Attachment I
Validating a Quantitative Real-Time PCR Method to Detect Dermo (*Perkinsus marinus*) in Texas Oysters

Contract number #1004831018
TWDB comments to Final Report

The study report reflects the tasks outlined in the scope of work.

REQUIRED CHANGES

General Draft Final Report Comments:
The study report provides a nice overview of the methodology and results as well as justification for sampling dermo infection in oyster populations and the need for developing the quantitative PCR method to improve sampling efficiency. Overall, the report is well-written aside from minor editorial mistakes which should be corrected prior to developing a final report.

Specific Draft Final Report Comments:

1. *Throughout*: Please consider adding page numbers to the report.
2. *Introduction, Page 2, last sentence of second paragraph*: Please spell out the acronym 'RFTM' the first time it is used. Please also review all other acronyms used in the report and similarly spell out the acronym the first time it is used.
3. *Objectives of Current Study, first paragraph*: Please consider numbering the objectives.
4. *Objectives of Current Study, second paragraph*: Please replace "..TWDB has been fortunate to have had access to TPWD's Coastal Fisheries.....for freshwater inflow assessments." to read ".. the state agencies have been fortunate.....". Freshwater inflow studies conducted by the tri-agencies have all benefited from TWPD's monitoring program.
5. *Methods, Overview, first paragraph*: Please clarify if the RFTM is "Ray's Fluid Thioglycolate Methodology" or "Ray's Fluid Thioglycollate Medium" as RFTM is used to indicate both. Additionally, please clarify the spelling of thioglycolate.
6. *Methods, QPCR Methods, second paragraph*: Please consider explaining meaning of the QPCR results based on the threshold-cycle of DNA amplification. This explanation may be more appropriate in the Results section, where it should clarify why high QPCR values correspond to low levels of dermo detection.
7. *Results, RFTM:QPCR Comparison of all Samples, second paragraph*: There seems to be a large spread of QPCR values spread about the regression line. Please address how QPCR values can be assigned to an infection category with confidence. For example, the report suggests that "very light levels of infection" correspond to QPCR 33 – 37.9; however the spread of QPCR values ranges from 22 – 40, thus encompassing levels ranging into "moderate infection".
8. *Results, San Antonio Bay Comparisons, second paragraph*: Please explain the consequences of freezing the oyster tissues to the study.

9. *Results, Individual Reef Comparisons, first paragraph*: Please explain why Fisher's Reef samples with a score of 0.0 Mackin Scale were omitted from Table 20; and similarly, please explain why duplicate samples were included in Table 21.

Figures and Tables Comments:

1. *Methods, Sample Collection*: Please consider adding a table to this section of the report that lists the oyster reefs sampled, their reef code (as used in the study), estuary and/or bay location. Or, consider adding the reef the code to Figure 1.
2. *Figure 1*: Panther Reef, Chicken Foot Reef, and Middle Ground Reef are not located on the map.
3. *Figure 2*: Please clarify the difference between figures (a) and (b) in the figure caption.
4. *Figure 11*: Please consider presenting the confidence intervals in another way so that the actual trend lines can be more easily seen.
5. *Figures 14 and 15; 16 – 19; and 20 – 23*: Please consider labeling each graph with the corresponding figure number or labeling the composite as a figure with each graph separately noted as (a), (b), (c), etc.