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Screening Ocean Samples from Georgia for the Presence of MSX and Dermo Using PCR and qPCR Methods

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INTRODUCTION

Two prevalent oyster parasites are that of MSX and Dermo. MSX, scientific name *Haplosporidium nelsoni* was first discovered in 1957 in the Delaware Bay as the causative agent for 90-95% of oyster deaths in that area that year (Ewart & Ford). It was later found in the Chesapeake Bay as well, and although it dominates in these areas it is known to frequent the entire Eastern coast. Dermo, scientific name *Perkinsus marinus* was originally found on the Gulf of Mexico and was originally classified as a fungus before being reclassified as a parasite in 1978. Dermo is known to frequent the entire East coast, as well as the Gulf of Mexico down the coast to Venezuela *(Oyster-Diseases-CB.Pdf)*. Currently both MSX and Dermo are leading causes in oyster populations along the East Coast, and have cost millions of dollars in losses to the oyster industry since their discovery. Both parasites frequent not only natural populations, but cultivated ones as well. Although they serve no threat to human populations, they are detrimental to the oyster populations that are used for food (Ewart & Ford).

This study aimed to track the population of both MSX and Dermo from ocean water samples collected at the Skidaway Estuary in Georgia. Ocean samples were collected from February-August of 2021 and sent to the Harvey Phyto Lab at the University of New Hampshire where DNA extractions were performed on them. This was followed by PCR methods to track which dates showed a presence. From there qPCR methods were performed to track the actual concentrations of Dermo in the original samples. These concentrations were then analyzed against salinity, pH, temperature, oxygen, and chlorophyll levels which were all taken at the time of sampling in order to show any strong correlations. This was the first study to look at the abundances of these diseases from Georgia using genetic techniques over a seasonal time scale.

Importance

The results of this study showed that MSX was present mainly during the months of June-August, whereas Dermo was present consistently, peaking in the months of April-May. This shows a seasonality of infection, and in turn shows when the best time of the year to induce treatments would be once a treatment is found. This could ultimately help prevent the losses oyster farms face along the East Coast, and help save natural oyster populations as well. These results are also interesting when compared to the spawning times of oysters in Georgia, which is from April-September. This study was able to show peak concentrations for Dermo in April-May which implies that Dermo could be infecting at the larval stage of development (Print, n.d.). Future research could look into trends in the following year in order to further confirm these results. Other possible studies could be done using the same methods in another location along the coast to compare differences in peak concentrations and overall presence. Further studies would also be necessary to prove that Dermo infects oysters at the larval stage.

MATERIALS AND METHODS

Table 1: Expanded PCR Specifications

PCR Component	Relevant Characteristics
Primers	
HnPm-A	5' AGC CAT GCA TGT CTA AGT ATA A 3'
HnePsp-B	5' GAT GTG GTA GCC GTT TCT CAG G 3'
Cvi 6-13 PCR Program	Initial 94°C for 2 minutes 30 cycles of: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 15 seconds Final: 72°C for 5 minutes
Will57 PCR Program	Initial 95°C for 2 minutes 23 cycles of: 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds Final: 72°C for 5 minutes

Primers were mixed at a 10uM concentration from the HnPm-A and HnePsp-B primer stocks that were originally 100uM. They were diluted in ultra-pure nuclease free water, and were able to be mixed with no primer dimers forming.

Sample Collection was done within the Skidaway River Estuary (SRE), Savannah Georgia. Weekly surface water samples were collected from February – August 2021 via a 5 L Niskin bottle. Sample water was then gently transferred to a 10 L carboy, and brough back to the laboratory for analysis. Additionally, water quality data was collected from the SRE using a YSI (Xylem Inc.) Professional Series Plus and Pro DDS probes. Physical water data parameters collected included temperature (°C), salinity (PSU), pH, and oxygen (mgl-1 and % DO)

Filtering for DNA was done for each water sample, three 47 mm polycarbonate filters were filtered in triplicate, across three size fractions: 0.2, 20, 100 µm. To achieve the higher size fractions, water was prefiltered through either 20 or 100 µm nitex mesh, and then filtered. All water was filtered under gentle pressure, and then immediately stored at -80°C until processing.

DNA extractions were performed using the Qiagen DNeasy Blood & Tissue Kit with minor modifications to the manufacturer's protocol. First, rather than pure ethanol, Fisher Chemical's Reagent Alcohol was used for step 3.. As all samples were on filters, , the first few steps also needed to be adjusted as follows. First all extraction chemicals were added directly to tubes containing the individual filter, taking care to be sure the solutions were added to the center of the filter to help wash the filter. Second, a second step was added post PBS buffer, proteinase K, AL bugger, and ethanol steps, where the filters were carefully removed with tweezers sterilized with ethanol . Then the liquid remaining in the tubes was translocated into the spin columns via pipette. The remaining steps were completed as per the given kit protocol, without repeating the final step as the 200uL of DNA extraction were sufficient for the remaining steps. The Qubit was also used to ensure each extraction had at least 0.01ug/mL of DNA present in the sample.

PCR Master Mix contained for each one sample being run 4.5uL of ultra-pure nuclease free water, 0.5uL of 4mM spermidine, 1uL of 0.5mg/mL BSA, 1uL of Primer mix, and 10uL of 2X OneTaq. From this, 17uL of master mix were combined with 3uL of DNA extraction sample in Template III PCR plates capped with the rubber cover accompanying them. Once placed in the

thermocycler another rubber barrier was added on top of the cap in order to prevent the lid directly touching and melting the plate. They were then ran on the thermocycler using the Cvi 6-13 PCR program.

E-gels were then used to visualize PCR products. E-gels were removed from their packaging and plugged into the Mother E-base. 15uL of E-Gel™ Low Range Quantitative DNA Ladder was added into the "M" lanes labeled for the marker. 15uL of each PCR product were then added into the lanes without loading buffer . Up to 48 samples could be run at one time. Once loaded the Mother E-base was plugged in and the gel ran for 23 minutes. Once done E-gels were moved to the blue light transilluminator where a picture was taken using a gel imager. Pictures were then labeled electronically, and analyzed to check off whether or not MSX and/or Dermo were present (*See Table 2*).

qPCR Master mix contained for each sample 25uL 2X OneTaq, 1.3uL of 4mM spermidine, 4uL Primer mix, 2.5uL 0.5mg/ml BSA, 2uL of competitors of different set concentrations, and ultra-pure nuclease free water to fill to 45uL of cocktail for each sample. The competitors were used in a 1:2 dilution series of 0.5; 0.25; 0.125; 0.0625; and 0.03125 pg/uL. One master mix was made for each competitor dilution, for a total of five master mixes. 5uL of DNA sample was added to 45uL of each master mix in TempPlate III PCR plates capped with the rubber cover accompanying them. Once placed in the thermocycler another rubber barrier was added on top of the cap in order to prevent the lid directly touching and melting the plate. They were then ran on the thermocycler using the Will57 PCR program.

Criterion Gels were then used to visualize the qPCR samples. Criterion gels were removed from their packaging and put into a gel electrophoresis chamber where 1X TAE buffer was then filled to the fill lines given on the chamber. Combs were removed, and using a syringe bubbles were removed and combs were straightened if necessary. 5uL of 50bp Ladder was added into lane 11 of the gels, and 10uL of qPCR products were added into lanes 1-10 and 12-26 of the gels. They were then placed in a gel electrophoresis chamber filled with 1X TAE buffer, and they were then run for 1 hour at 150 volts. Once completed the gels were removed from their plastic casing, using DI water to help make them more slippery and less likely to rip. They were then placed in a Tupperware container with originally 15uL of 10,000X SYBR Safe in 300mL of distilled water, adding 4uL of fresh SYBR Safe for each gel to account for light sensitivity of the reagent. The gels were then left in there for 5 minutes while being slightly agitated as they soaked up the reagent. They were then removed and placed into another piece of Tupperware containing DI water in order to rinse off excess SYBR safe stain. From there they were placed onto a blue light transilluminator, where a picture was taken using a gel imager. Pictures were then labeled electronically for future analysis.

Criterion Gel Analysis The fluorescence band denoting DERMO was analyzed from each Criterion gel using ImageJ. From each sample, the area integrated intensity and mean grey value were measured, additionally these metrics were gathered from a region of the image with no fluorescence to serve as the background intensity. For each measurement, the corrected total cell fluorescence (CTCF) was calculated using the following equation: CTCF = integrated density –

(area of selected cell x mean fluorescence of the background). A standard curve between the CTCF and the competitors was created, and a linear regression was used to find an explanatory equation for that relationship. That equation was then used on the unknown samples to calculate a DERMO concentration of the sample (pg DNA μl^{-1}).

RESULTS

Table 2: E-gel Analysis

DERMO

	100 µm			20 µm			0.2 µm		
Date	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
Feb 26									
March 12									
March 19									
March 25									
April 2									
April 9									
April 16									
April 23									
May 3									
May 14									
June 9									
June 25									
July 16									
August 1									
August 14									
August 21									
August 28									

MSX

	100 µm			20 µm			0.2 µm		
Date	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
Feb 26									
March 12									
March 19									
March 25									
April 2									
April 9									
April 16									
April 23									
May 3									
May 14									
June 9									
June 25									
July 16									
August 1									
August 14									
August 21									
August 28									

Table 2: Based on the E-gel visualization results, this table was made in order to organize which dates were positive (P) or negative (N) for MSX and Dermo so they were easier to compare than from the original pictures.

Figure 1: Dermo Concentration Over Time

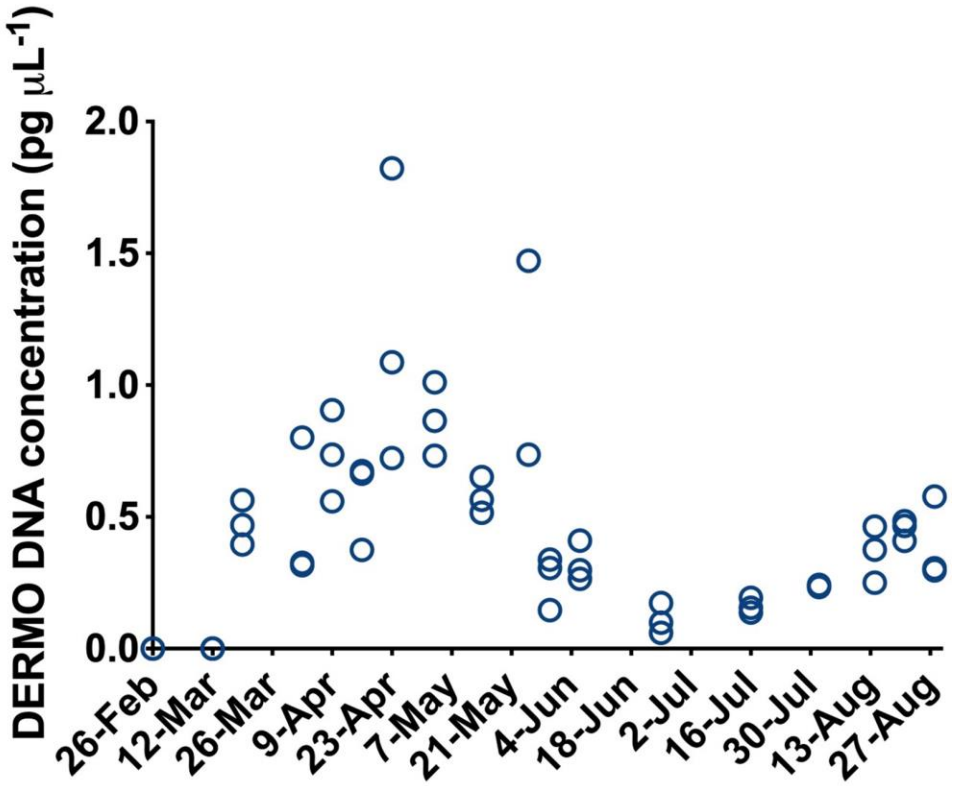

Figure 1: Based on the ImageJ analysis of the Criterion Gels, three concentrations of each raw sample were calculated and plotted by date in chronological order.

Figure 2: Dermo Concentration vs. Temperature

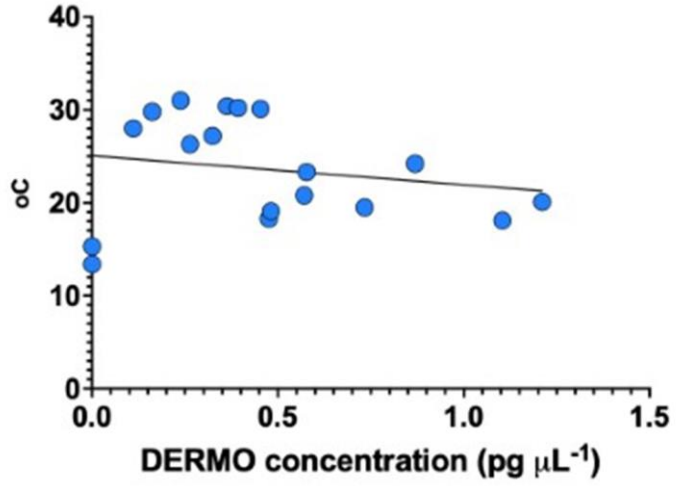

Figure 2: The temperature (in Celsius) of the original water samples was plotted against the calculated concentrations in order to see if there was any correlation between the two variables.

Figure 3: Dermo Concentration vs. Salinity

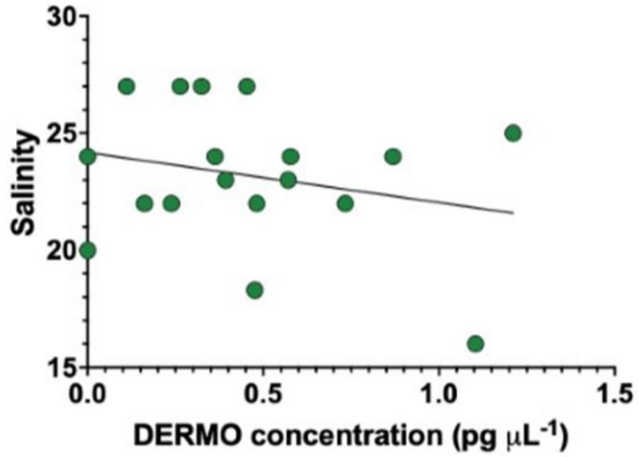

Figure 3: The salinity of the original water samples was plotted against the calculated concentrations in order to see if there was any correlation between the two variables.

Figure 4: Dermo Concentration vs. pH

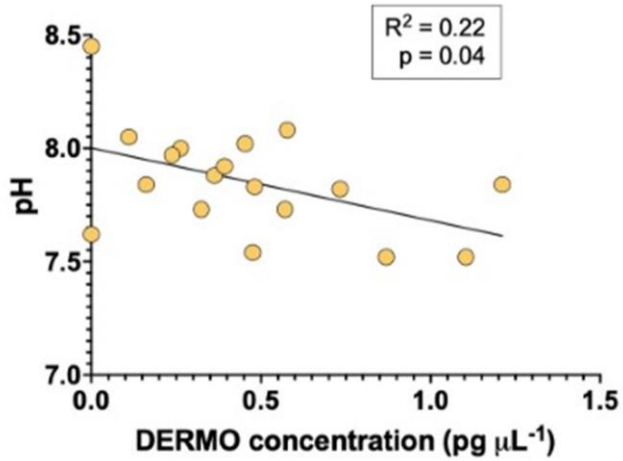

Figure 4: The pH of the original water samples was plotted against the calculated concentrations in order to see if there was any correlation between the two variables.

Figure 5: Dermo Concentration vs. Oxygen levels

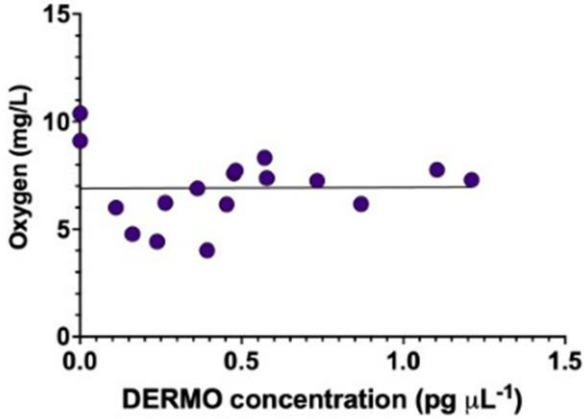

Figure 5: The oxygen levels in the original water samples was plotted against the calculated concentrations in order to see if there was any correlation between the two variables.

Figure 6: Dermo Concentration vs. Chlorophyl

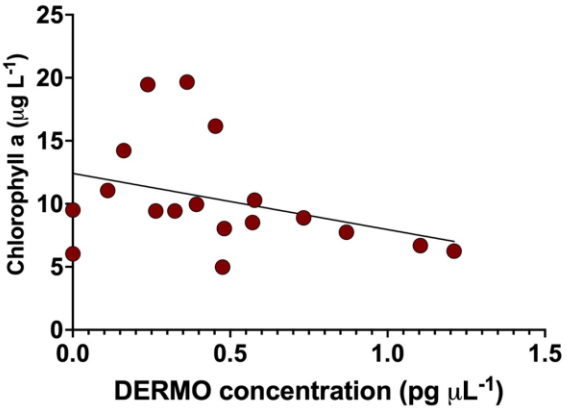

Figure 6: The chlorophyll levels in the original water samples was plotted against the calculated concentrations in order to see if there was any correlation between the two variables.

DISCUSSION

In Table 2 a clear difference can be seen between the presence of MSX and Dermo in the samples over time. Dermo appears to be present fairly consistently throughout the dates, excluding February 26th. Whereas MSX was present mainly during the months of June-August. MSX was also shown to be present in some of the 0.2um samples from March-April. This could be due to the size of the MSX parasite, but further research would be required to know for sure why this occurred.

In Figure 1 it is shown that although Dermo was present consistently over the months, it was present at varying levels. Dermo appears to peak in the spring months of April-May, with another small peak at the end of August. These peaks do match the twice-yearly recruitment peaks of oysters in the Skidaway River Estuary. This could mean that if fall months past August were sampled that there could have been another peak in the fall. This could be a potential area for future research.

In Figures 2, 3, 5, and 6 no strong associations can be seen between the Dermo concentration and the temperature, salinity, oxygen, or chlorophyll levels. This shows that Dermo does not have a strong preference over any of these variables, and would be able to infect at a large variety for each. This is initially surprising due to the lack of Dermo present during the February 26th date, as one might assume temperature would have had an effect on the parasite concentration. However it is possible that the reason for there being no correlation is not because the parasite prefers a certain water temperature, but rather the oysters do. It is possible that in the winter month of February there were no oysters present to infect, and that is why there was no Dermo present.

In Figure 4 a strong negative association can be seen between the pH and the concentration of Dermo. This seems to indicate that Dermo prefers to live at a lower pH rather than a high one. This would mean that an oyster from a lower pH ocean would be more likely to be infected by higher levels of Dermo. Future studies into levels of Dermo in higher vs. lower pH waters could be looked at for future experimentation as well.

References

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