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2012 Summer/Fall MSX Prevalence Report in the Damariscotta River Estuary, ME

Nicole Messerman & Tim Bowden

ABSTRACT: The Eastern oyster, *Crassostrea virginica,* makes up an important commercial fishery along the East and Gulf coast of North America. Consumer demand has permitted the development of the oyster aquaculture industry. Specifically, about 70% of the total production of harvested oysters in Maine came from the Damariscotta River Estuary in 2010(DMR). Unfortunately, disease issues have become a major concern. One particular disease affecting the eastern oyster is known as MSX (multinucleated sphere unknown). MSX is caused by the protozoan parasite, *Haplosporidium nelsoni*, and has devastating effects on cultured and wild oysters, killing 90-95% of the oysters it infects. Until recently, MSX had not managed to reach epizootic levels in Maine. However, in the summer of 2010 MSX was responsible for significant mortality among cultured oysters in the Damariscotta River estuary and mortalities have not abated since. This study investigated the current prevalence of MSX in one commercial oyster site and two natural oyster beds located in the Damariscotta river estuary by using histology and PCR-based assay specific for *H. nelsoni*. Over a 3-month period (August-October 2012), a total of 316 oysters were surveyed. *H. nelsoni* was prevalent at every site. At some sites prevalence was as high as 50%. These results are similar to previous prevalence surveys and suggest that MSX disease has not subsided in the Damariscotta River estuary.

Introduction

The Eastern oyster, *Crassostrea virginica,* makes up an important commercial fishery along the East and Gulf coast of North America. Consumer demand has permitted the development of the oyster aquaculture industry. In Maine about 70% of the total production of harvested oysters came from the Damariscotta River Estuary in 2010 (DMR). Unfortunately, disease issues have become a major concern.

MSX (multinucleated sphere unknown) disease, caused by the parasite *Haplosporidium nelsoni,* has devastating effects on cultured and wild oysters, killing up to 90-95% of the oysters it infects. It was first discovered in the spring of 1957 in Delaware Bay and a few years later in the Chesapeake Bay. Since the discovery of the parasite, it has expanded its range as far north as Canada (Stephenson et al. 2003).

The complete life cycle of the parasite remains unknown making the parasite difficult to manage. Proposed life cycles have been published (Farley 1967, Couch et al. 1966) but because the disease has not been successfully transmitted from infected oyster to naïve oyster the infective stage is still unknown. With concerns that MSX was moving into Maine's oyster industry, a survey was performed in 1990 measuring the prevalence in the Damariscotta River Estuary, ME. At this time, results showed that MSX was not at epizootic levels (Sherburne and Bean 1991). In the summer of 2010, MSX was again identified in commercial operations in the Damariscotta River Estuary. This resulted in significant mortality among the cultured oysters in the river and has not abated in intensity.

It is important to continue monitoring the prevalence and infection intensity of MSX in the Damariscotta River Estuary. With the changes in the environment and changes in disease resistance over time, it is necessary to survey MSX in river to compare the disease intensity over time. In this report, the prevalence of MSX was surveyed in one commercial site and two natural beds located in an area that is highly concentrated with oyster farms in the Damariscotta River, ME.

Methods

Oyster collection

Between 30 and 40 oysters (*Crassostrea virginica*) were randomly sampled by a member of Pemaquid Oyster Company. Oysters were collected from 3 sites within the Damariscotta River Estuary, ME at three different time points in late summer into fall 2012 (Figure 1). Two natural beds and one commercial site were surveyed. Oysters provided by Pemaquid Oyster were from 2010 MSX non-resistant seed. The 316 oysters collected in total over the course of 3 month surveyed (August, September, October 2012) were screened for the parasite *Haplosporidium nelsoni* (MSX) using both histology and Polymerase Chain Reaction (PCR).

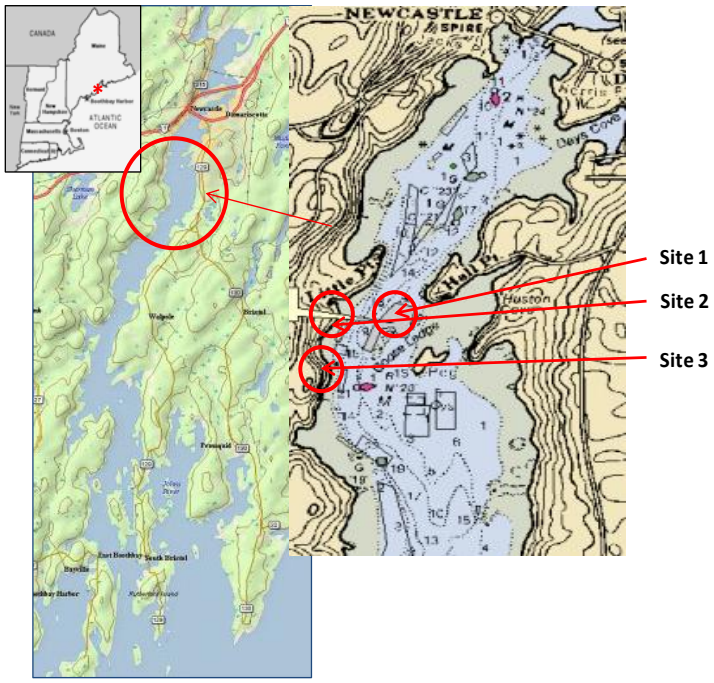


Figure 1. Map of sample locations in the Damariscotta River Estuary, Maine, USA. Sites include: Commercial (Pemaquid Oyster Company (site 1)), natural bed A (site 2), and natural bed B (site 3).

Preparation for histological examination and genetic screening for the parasite

Oysters were shucked by separating the two valves at the hinge using a shucking knife and cutting the adductor muscle away from one of the valves. Gill sample were taken from each animal for PCR analysis. In between each oyster, dissecting tools and the shucking knife were disinfected and cleaned with a 5% bleach solution and then rinsed in 70% ethanol (EtOH). Gill tissue samples were preserved in 1.5 ml

microcentrifuge tubes with 95% EtOH and stored at 4°C. Three days later the EtOH was replaced with new 95% EtOH. After, the whole oyster was removed from the shell and a specific cross section of the visceral mass was made using a disposable scalpel. A new scalpel was used for each animal. This cross section included part of the gills, mantle, reproductive tissue, stomach, intestines and digestive gland. The section was placed in the corresponding cassette. Two cross sections were placed into each cassette in the same orientation. Sections were fixed in Davidson's fixative (2 parts 37% Formalin, 3 parts 95% EtOH, 1 part glacial acetic acid, and 3 parts tap water) for 24 hr and then replaced with 70% EtOH. The University of Maine Animal Health Laboratory prepared the histological slides. Briefly, the tissue for histological sections were embedded in paraffin, sectioned and processed using standard methods and then stained with hematoxylin and eosin.

DNA extraction and amplification by PCR

DNA from the gill tissue samples, collected and stored in 1.5ml microcentrifuge tubes with 95% EtOH, was extracted using the Chelex protocol as described by Aranishi and Okimoto (2006). Between 5-7 mg of tissue was weighed and then resuspended in 100 µl Chelex Buffer (4 M Urea, 1 µl Proteinase K, 5% Chelex, 1% Nonidet P-40 substitute, 1% Tween 20). Chelex buffer was preheated to 60°C before adding to the tissue. The samples were then incubated at 55°C for 1 h. The samples were vortexed at the start of incubation and 30 minutes into incubation and at the end of 1 h. After, the samples incubated for 8 min at 100°C. The samples were then centrifuged for 5 minutes at 15,000g to pellet the remaining tissue. 100 µl of the supernatant was transferred to a new 1.5 ml tube where 1 µl 100TE buffer (pH 8.0), 50 µl Ammonium acetate ($AcONH_4$) and 400 µl 95% EtOH was added to each tube. The samples were vortexed thoroughly on high and then centrifuged for 20 minutes at 15,000g to pellet the DNA. The supernatant was removed leaving the DNA pellet. The pellet was washed with 200 µl of 70% ice cold EtOH and centrifuged for 5 minutes at 15,000g. This was repeated 2 more times for a total of 3 washes. The pellet was air dried at room temperature and resuspended in up to 100µl 10T0.1E buffer. The size of the pellet determined the amount of 10T0.1TE buffer added for resuspension. For large pellets 100 µl buffer, for medium pellet 50 µl and for small pellets 20 µl buffer.

DNA concentrations (µg ml^{-1}) were determined using a spectrophotometer (Biowave II, Biochrom, UK; Tray Cell Hellma Analytics). The 260:280 nm absorbance ratio was used to determine the purity of the sample. This was between 1.5 and 2.0 for all the samples assayed. DNA extractions were

stored at -20^{o}C. Since genetic screening was used alongside histology as a yes or no confirmation of the parasites presence, the DNA concentrations for each sample was not adjusted to any uniform concentration.

The *H. nelsoni* specific PCR forward primer MSX A' described by Day et al. 2000 (Day et al. 2000) and the reverse primer MSX B originally described in Stokes et al. 1995 (Stokes et al. 1995) were used in this assay. MSX- A' (5'-CGACTTTGGCATTAGGTTTCAGACC-3') and MSX-B (5'-ATGTGTTGGTGACGCTAACCG-3') primers amplified a 572 bp sequence of the small subunit ribosomal RNA (SSU rRNA) gene from *H. nelsoni* present in oyster tissue. A PCR master mix was made containing 5x Green GoTaq Flexi buffer, $MgCl_2$ (25nM), dNTP master mix (10mM), GoTaq Hot Start Polymerase (5 µ/µl), Nuclease free water, and forward and reverse primers (10 mM). 1.5 µl of DNA template was added to each reaction making the final volume of each reaction 25µl. The reactions were cycled in a Thermal cycler (Eppendorf) for 5 minutes at 94^{o}C and 30 cycles of 94^{o}C for 60 seconds, 60^{o}C for 50 seconds, 72^{o}C for 29 seconds followed by 72^{o}C for 29 seconds and then held at 4^{o}C until stored in -20^{o}C. Each PCR reaction was checked for product amplification using a 1.3% agarose gel with ethidium bromide. A 100bp ladder (NEB) with 6x loading dye was run as a marker. The gels were run at 85V for 65 minutes and photographed under UV light (U:Genius, Syngene, UK)(fig. 2-4). Prevalence (as a percentage) based on PCR results were calculated as;

$$\frac{\text{number of MSX positive oysters}}{\text{total number of oysters examined.}} \times 100$$

This was calculated per site at each of the time point surveyed.

False negative control by PCR

To make sure that there was no inhibition and that DNA extractions were successful a subset of samples that were found to be negative for MSX through PCR were re-run using 16S mitochondrial rRNA *C. virginica* primers (forward primer 5'-AGTACCTGCCCAGTGCGACAATAA-3' reverse primer 5'-TCGAGGTGCCAAGCCCTTTAGTTA-3'). The reaction cycle was for 5 minutes at 94^{o}C and 30 cycles of 94^{o}C for 60 seconds, 60^{o}C for 50 seconds, 72^{o}C for 29 seconds followed by 72^{o}C for 29 seconds and then held at 4^{o}C until stored in -20^{o}C.

Histological examination

Stained sections were screened for the presence of *H. nelsoni* using an Olympus BX51 light microscope (fig. 5-8). The MSX infection intensity rating used in this study was adapted from the *Haplosporidium nelsoni* (MSX) rating system Haskin Shellfish Research Laboratory, Rutgers University. Infection intensity scored from 0 to 5 (Table 1). The 316 animals sectioned were first scanned quickly over the whole body at 40x magnification and then quickly at 100x magnification starting at the gills and working over the entire body (visceral mass, mantle). After the whole animal was scanned at the lower magnification the gills were then examined thoroughly at 400x magnification and scored based on Table 1. The intensity of infection found in the gills determined how in-depth the examination of the body was. Once the gills were examined and scored the body was then examined at 400x magnification and scored based on Table 1. For every oyster, the gills and body were scored individually and then the animal was given an overall infection intensity score based on the infection intensity found in both the gills and the body. Prevalence (as a percentage) based on histology results were calculated the same as the PCR results.

Table 1.

Table 1. MSX Rating Score	Infection Intensity	Parasite Count
0	None detected	No parasites found in the section
1	Rare	1-10 parasites in entire animal
2	Very light	11-100 parasites in entire animal
3	Light	More than 100 parasites in entire animal
4	Moderate	Averaging 1-5 parasite in every field of view (400X)
5	Heavy	Averaging more than 5 parasites in every field of view (400X)

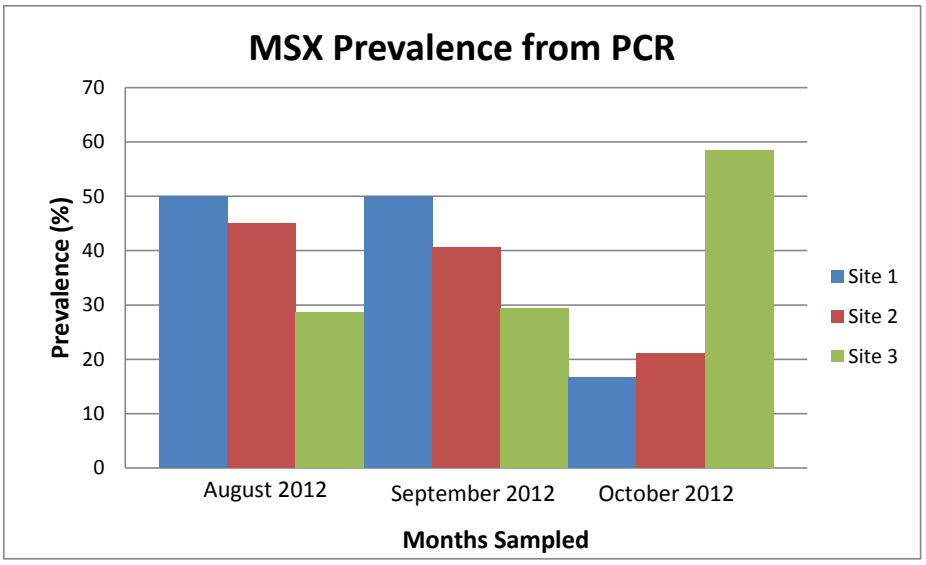
Results/Discussion

MSX was present at all sites surveyed. Out of the three sites, the overall highest prevalence was observed at the commercial site (site 1). In August and September the prevalence remained high and by October it had decreased greatly. The prevalence in natural bed A (site 2) gradually decreased over the three months surveyed while the opposite was observed in the natural bed B(site 3), the prevalence increased over time and spiked in October. Overall the MSX prevalence determined using PCR was greater than the prevalence found using histology. This is common since PCR is a more sensitive tool but the difference in prevalence between the two methods was not a great as expected and this could be due to mis-diagnosis through histology. *Haplosporidium costale* is another oyster parasite that is found in oysters in the Damariscotta River (causing SSO disease) and it looks very similar to *H. nelsoni*. It is not possible to distinguish between the two parasites with histology. So negative PCR results with a positive histology result could suggest that there was incorrect parasite identification and further testing using SSO primers is necessary.

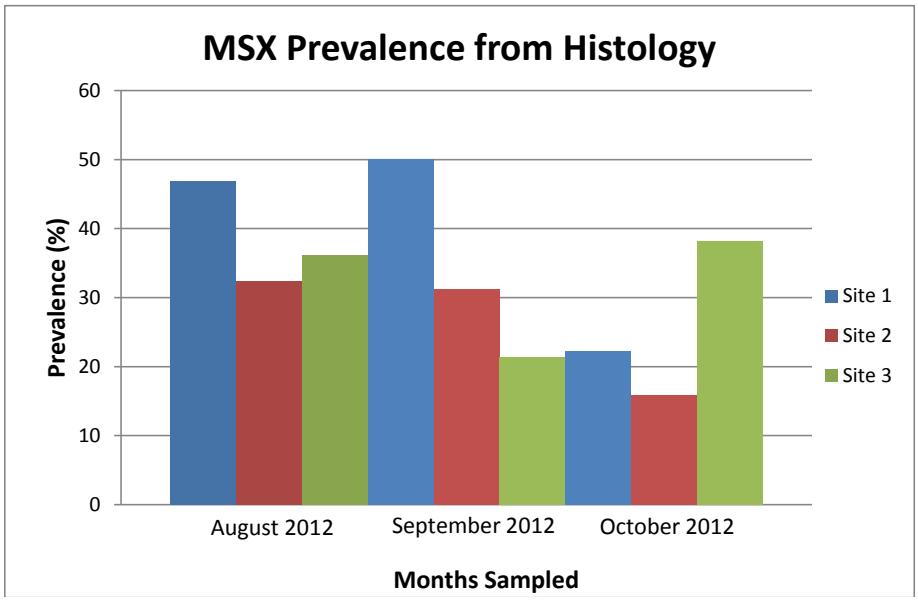
Prevalence (see appendix 1 and graphs) at each site on the Damariscotta River were as follows: August 2012 Pemaquid Oyster Company PCR: 50% (16/32) histology: 46.88% (15/32), natural bed A PCR: 45% (18/40) histology: 32.43% (12/37), natural bed B PCR: 28.57% (10/35) histology: 36.11% (13/36). September 2012 Pemaquid Oyster Company PCR: 50% (16/32) histology: 50% (6/12), natural bed A PCR: 40.63% (13/32) histology: 31.25% (10/32), natural bed B PCR: 29.41% (10/34) histology: 21.43% (3/14). October 2012 Pemaquid Oyster Company PCR: 16.67% (6/36) histology: 22.2% (8/36), natural bed A PCR: 21.05% (8/38) histology: 15.79% (6/38), natural bed B PCR: 58.33% (21/36) histology 38.24% (13/34).

Conclusions

Based on the results of this small prevalence study, it is apparent that MSX infection has not subsided in the Damariscotta River Estuary and it is just as prevalent as in past surveys (Spring 2011 DMR survey). Although this study surveyed a small area, it provides a representation of the levels of MSX present within an area that is heavily concentrated with commercial oyster farms within the river.



Graph 1. MSX prevalence at 3 sites sampled in the Damariscotta River Estuary from August through October 2012. Prevalence calculated from positive PCR. For each site: number of MSX positive oysters from PCR divided by the total number of oysters multiplied by 100.



Graph 2. MSX prevalence at 3 sites sampled in the Damariscotta River Estuary from August through October 2012. Prevalence calculated from positive histology. For each site: number of MSX positive oysters from histology divided by the total number of oysters multiplied by 100.

Gel electrophoresis images:

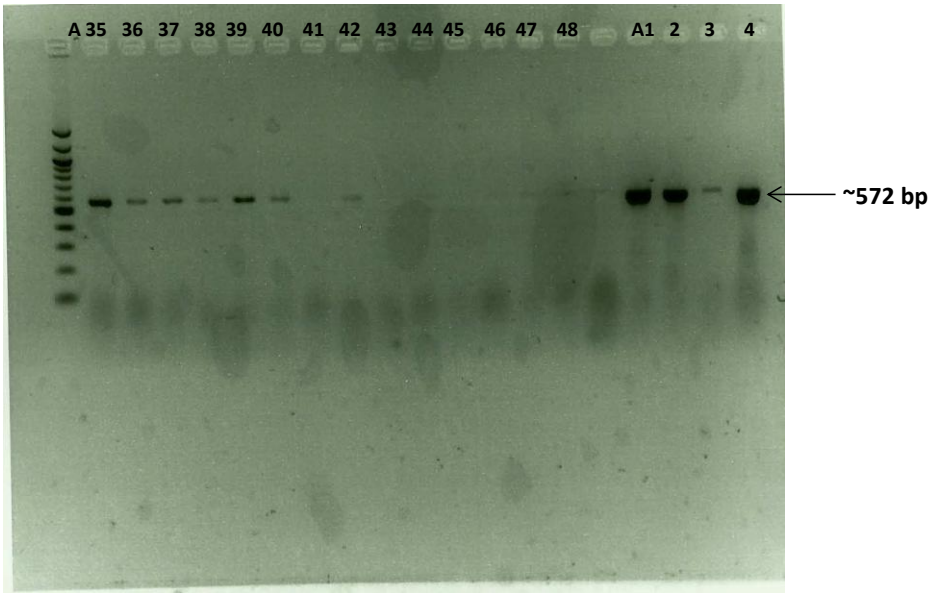


Figure 2. 1.3% agarose TBE gel. Lane 1 100 bp ladder, lanes 2-20 individual oysters collected August 2012. A1-4 commercial (site 1) and A35-48 natural bed A(site 2). Positive bands for *H. nelsoni.*

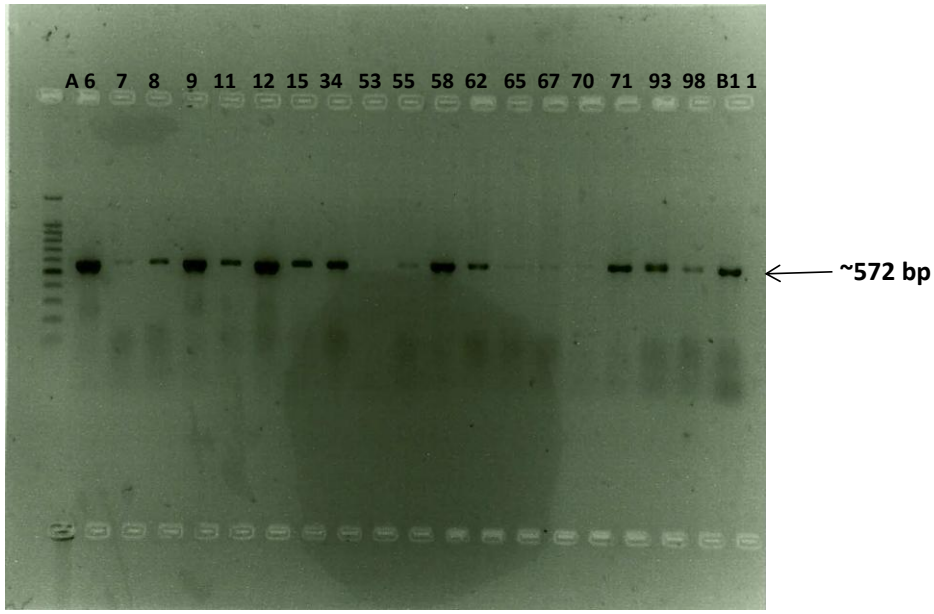


Figure 3. 1.3% agarose TBE gel. Lane 1 100 bp ladder, lanes 2-20 individual oysters collected August 2012 and September 2012. Mix of A6-15 commercial (site 1) and A34-71 natural bed A (site 2) A93 & 98 natural bed B (site 3) B1 1 commercial(site 1) collected in September 2012. Positive bands for *H. nelsoni.*

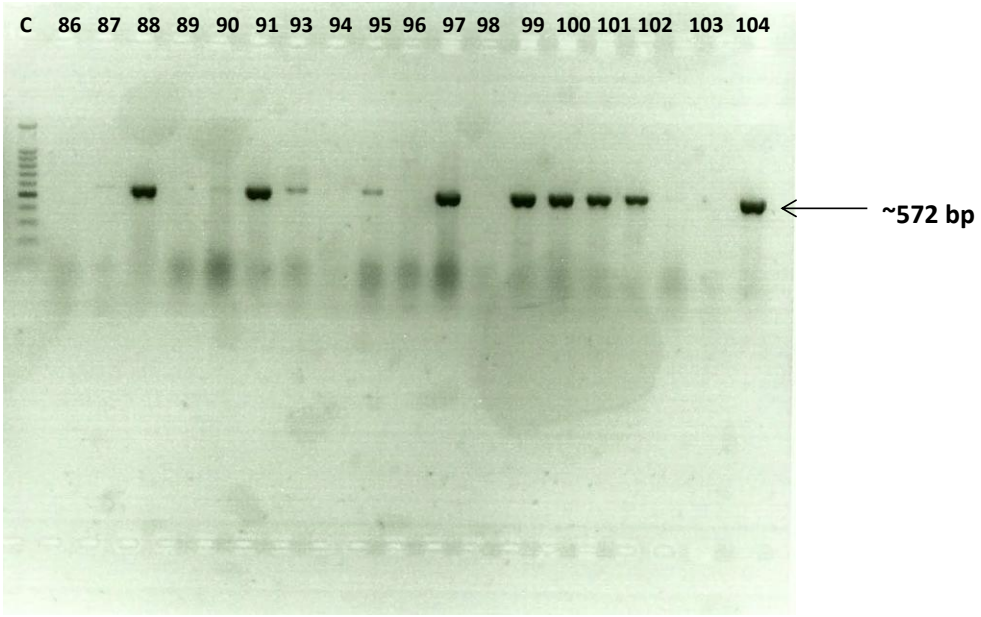


Figure 4. 1.3% agarose TBE gel. Lane 1 100 bp ladder, lanes 2-20 individual oysters collected October 2012. C86-104 natural bed B(site 3).Positive bands for *H. nelsoni.*

Histological images:
Levels of MSX intensity ranged from light to heavy with some oysters even having spores present (see appendix 1). Different intensities and stages of infection (figure 5-8):

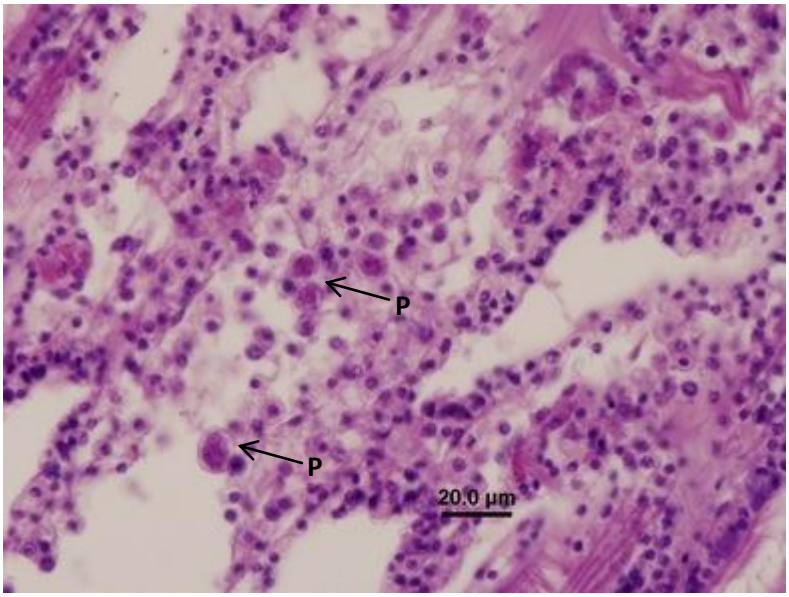


Figure 5. Heavy infection in wild oyster from natural bed A (site 2) collected August 2012. 400x magnification. *H. nelsoni* plasmodia (P) present within the gills.

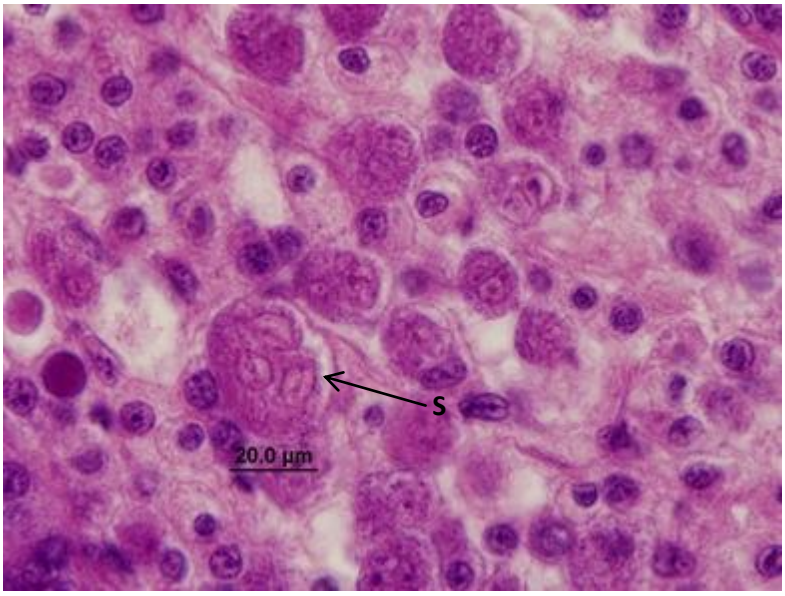


Figure 6. Heavy infection in wild oyster from natural bed B (site 3) sampled August 2012. 1000x magnification under oil. *H. nelsoni* spores (S) present within the body.

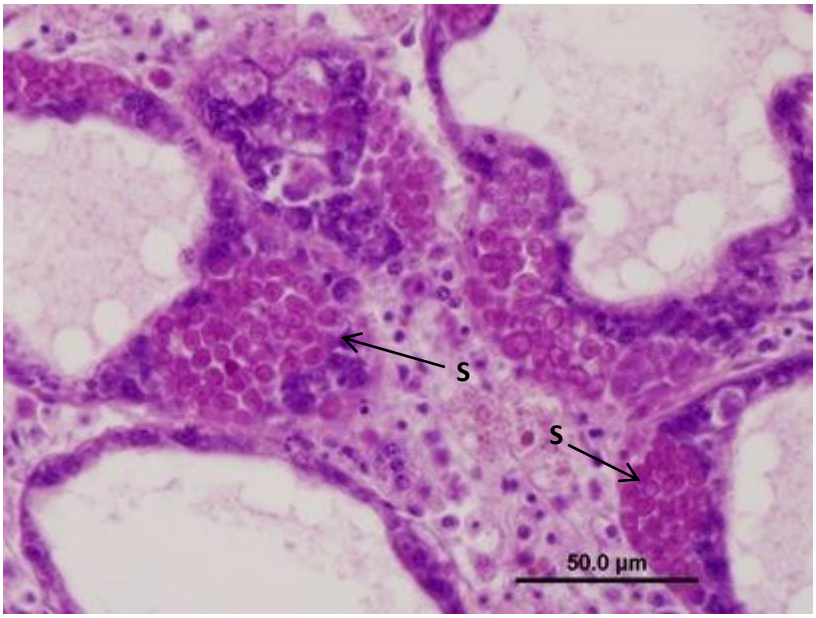


Figure 7. Heavy infection in commercial oyster (site 1) sampled September 2012. 400x magnification. *H. nelsoni* spores (S) throughout the body.

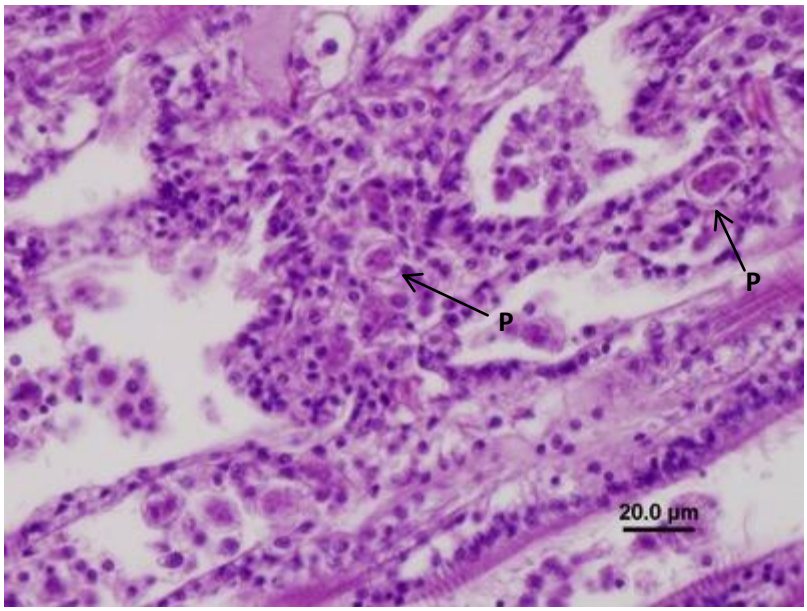


Figure 8. Light infection in wild oyster from natural bed A (site 2) sampled October 2012. 400x magnification. Plasmodia (P) present throughout the gills.

References

Aranishi, F.,Okimoto, Takane. (2006). A simple and reliable method for DNA extraction from bivalve mantle. *Journal of Applied Genetics*, **47**, 251-254.

Couch, J., Farley, A.C. & Rosenfield, A. (1966) Sporulation of Minchinia nelsoni (Haplsporida, Haplosporidiidae) in *Crassostrea virginica* (Gmelin). *Science,* **153,** 1529-1531.

Day, J.M., Franklin, D.E. & Brown, B.L. (2000) Use of Competitve PCR to Detect and Quantify *Haplosporidium nelsoni* Infection (MSX disease) in the Easter Oyster (*Crassostrea virginica*). *Marine Biotechnology,* **2,** 456-465.

Farley, A.C. (1967) A proposed life cycle of *Minchinia nelsoni* (Haplosporida, Haplosporidiidae) in the american oyster *Crassosstrea virginica*. *Journal of Protozoology,* **14,** 616-625.

Sherburne, S.W. & Bean, L.L. (1991) Final Progress Report on the Pathological Testing Program to Determine the Nature and Extent of the MSX (*Haplosporidium nelsoni*) Disease Problem in Damariscotta River American Oysters, *Crassostrea virginica*. In, Maine Departement of Marine Resources.

Stephenson, M.F., Mcgladdery, S.E., Mailet, M. & Veniot, A. (2003) First reported occurrence of MSX in Canada. Abstract. *National Shellfisheries Association*.

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

Appendix 1. Data