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NOTE

Haplosporidium nelsoni and *Perkinsus marinus* occurrence in waters of Great Bay Estuary, New Hampshire

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ABSTRACT: In Great Bay Estuary, New Hampshire, USA, *Haplosporidium nelsoni* and *Perkinsus marinus* are 2 active pathogens of the eastern oyster *Crassostrea virginica* (Gmelin), that cause MSX (multinucleated sphere with unknown affinity 'X') and dermo mortalities, respectively. Whereas studies have quantified infection intensities in oyster populations and determined whether these parasites exist in certain planktonic organisms, no studies thus far have examined both infectious agents simultaneously in water associated with areas that do and do not have oyster populations. As in other estuaries, both organisms are present in estuarine waters throughout the Bay, especially during June through November, when oysters are most active. Waters associated with oyster habitats had higher, more variable DNA concentrations from these pathogenic organisms than waters at a non-oyster site. This finding allows for enhanced understanding of disease-causing organisms in New England estuaries, where oyster restoration is a priority.

KEY WORDS: Protozoan pathogen · Estuarine water column · Eastern oyster · MSX · Dermo · QCPCR · Plankton

1. INTRODUCTION

Eastern oysters *Crassostrea virginica* are a foundation species in estuaries where they reside, providing sanctuary for juvenile marine life, buffering effects of extreme weather (Brandon et al. 2016), and removing nitrogen from the ecosystem via filter feeding (Newell et al. 2005). In Great Bay Estuary (GBE), New Hampshire, eastern oyster populations have declined dramatically (Beck et al. 2011). Research focused on restoration efforts in GBE (Grizzle et al. 2021) can be enhanced by examining reasons for eastern oyster decline, specifically the diseases multinucleated sphere with unknown affinity 'X' (MSX) (caused by *Haplosporidium nelsoni*) and dermo (caused by *Perkinsus marinus*). In 1995, an *H. nelsoni* epizootic event linked to increases in both temperature and salinity led to unusually high levels of oyster mortality in Piscataqua River (Barber et al. 1997). At the present time, both MSX and dermo disease are prevalent in GBE oysters (Eckert 2016). In 2018, MSX infections were found in roughly 10% of the population, and dermo infections in roughly 80% of the population (Patterson & Sullivan 2020).

To gain a better understanding of the distribution and etiology of both diseases in GBE, this study examined occurrence of *H. nelsoni* and *P. marinus* DNAs in water at a native oyster reef, an oyster farm, and a site lacking native oyster reefs or oyster farms, throughout oyster spawning and harvest season

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(June through November) using a molecular test (quantitative competitive PCR, or QCPCR) to simultaneously detect and quantify the disease agent DNAs. This accurate and economical method entails co-amplification of target DNA with known quantities of competitor DNA to allow for relative quantification of DNA from 2 different parasite loci without fluorescent labeling (Zentilin & Giacca 2010). Similar methodology was used for *H. nelsoni* (Day et al. 2000) and for *P. marinus* (Yarnall et al. 2000), in both cases producing specific and sensitive detection.

2. MATERIALS AND METHODS

2.1. Study location and sites

Great Bay is a well-mixed estuary located along the North American Atlantic coast near the southern portion of the Gulf of Maine in New Hampshire. Three study sites were selected based on known locations of oyster groups in GBE. Nannie Island had a native oyster reef that has been declining over recent years, was protected from harvest, and had a total oyster population of roughly ~60 000 oysters, situated on 3.6 ha bottom (https://www.nature.org/en-us/about-us/where-we-work/united-states/new-hampshire/oyster-restoration-in-the-great-bay-estuary/). The oyster farm situated in Little Bay had ~400 000 cultivated oysters on a leased bottom area of 4 ha. The reference site at Adams Point had no detectable oysters over a bottom area of 3.5 ha (Fig. 1). All sampling sites were ~2.5 m deep at high tide, had similar bottom type, and constituted ~10 m^2 area. Oysters deployed at the farm in this study were an MSX-resistant strain derived from the Haskin northeast high survival (NEH) diploid oyster obtained from Muscongus Bay Aquaculture. (Haskin & Ford 1979, Ford & Haskin 1987, Guo 2021).

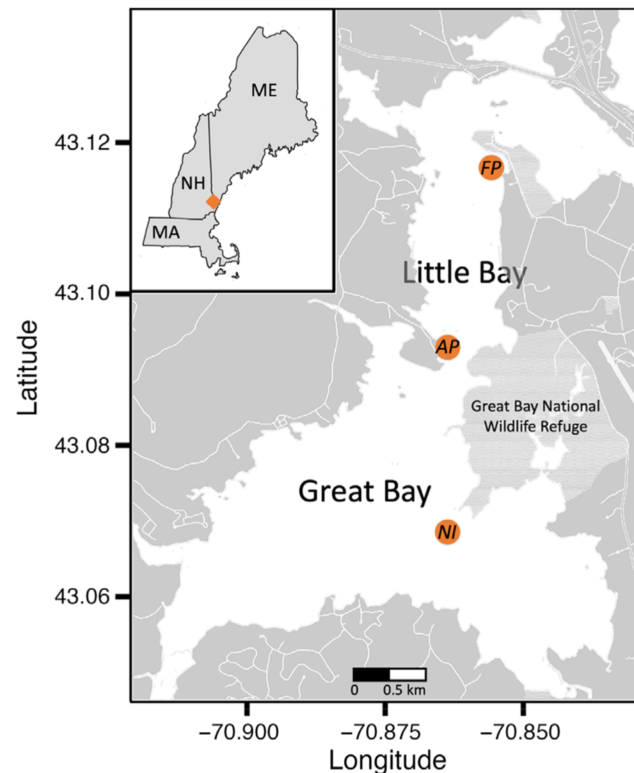

Fig. 1. Great Bay Estuary (with a regional inset of US New England states bordered in black). Sampling sites (orange points) included an oyster reef at Nannie Island (*NI*), an oyster farm near Fox Point (*FP*), and a site lacking a substantial oyster population between the other 2 sites near Adams Point (*AP*)

2.2. Samples

Water was subsampled from samples collected for a weekly survey of plankton from June through November 2020, except during instances of adverse weather or unsafe conditions (27 weeks, n = 78 water samples) (Stasse et al. 2022). All sampling occurred on the same day in the same order, except when conditions were unsafe to sample. Two replicate horizontal surface tows were made using a 64-µm mesh net (31 cm diameter) according to standard operating procedures laid out by the US Environmental Protection Agency (https://cfpub.epa.gov/si/si_public_record_report.cfm?Lab=NHSRC&dirEntryId=284802) and summarized briefly here. Tows were conducted ~0.3 m below the surface for approximately 2 min, resulting in ~38 m^3 of water sampled per tow. Due to strong and extreme tidal currents in GBE, sample volume was verified by use of a flow meter situated in the mouth of the net. Organisms and particles captured were preserved with formalin sucrose (Haney & Hall 1973) on board the vessel. Although formalin sucrose is known to fragment DNA, it was observed that the sizes of PCR products were sufficiently small as to not be adversely affected by the formalin.

2.3. DNA extraction and quantification

Each water sample was later 0.2-µm filtered (Pall™), and each filter was placed into a bead beating tube of the QIAGEN DNeasy PowerWater kit (GEN) for DNA extraction. Quantity and quality of

extracted DNA was determined using TapeStation™ Genomic DNA ScreenTape analysis protocol (Agilent). The concentration of each DNA sample was standardized to 5 ng μl^{-1} and used in QCPCR to determine relative amounts of *Haplosporidium nelsoni* and *Perkinsus marinus.*

2.4. QCPCR for detection and quantification of *H. nelsoni* and *P. marinus*

Each DNA extract was tested to ensure there was no PCR inhibition. All assays included positive and negative controls. Prior to QCPCR, detection of *H. nelsoni* and *P. marinus* DNA from water samples was accomplished using a single PCR reaction with primers HnPm-A and HnePsp-B (W. T. Brooks unpubl. data) (Table 1). Controls included DNA from algae in the vicinity (*Ulva* sp.) that did not amplify with primers (negative) and a DNA extract of whole oyster that previously had been diagnosed by histology to be infected by both MSX and dermo that amplified with the primer set (positive). For specimens showing one or both parasites in initial diagnostic reactions, subsequent quantification was performed. Five reactions, one for each competitor concentration, were prepared as 30 μl cocktails of 15 μl of *Taq*, 0.8 μl of 4 mM spermidine, 5 μl of 10 μM primer mix, 1.5 μl of 5 mg ml^{-1} BSA, 1.67 μl of one competitor, 5 μl (25 ng) of template DNA, and 1.03 μl of water. Thermocycling occurred with these parameters: 95°C for 2 min; 23 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec; and a final extension at 72°C for 5 min. This included addition of competitive DNA of similar base pair length (281 bp) to *H. nelsoni* and *P. marnius* amplicons (335 bp and 368 bp, respectively) previously generated using the HnPm-A and HnePsp-BC primers (W. T. Brooks unpubl. data) (Table 1). The competitor was serially diluted to the following concentrations: 0.250, 0.125, 0.025, 0.012 and 0.002 pg μl^{-1}, resulting in roughly logarithmic concentration steps. Controls for QCPCR were the same as the PCR controls. Amplification products were electrophoresed in adjacent wells of 5% Criterion™ TBE polyacrylamide gel (Bio-Rad), stained with GelRed Nucleic Acid Stain (Thomas Scientific), and visualized using a blue light transilluminator (ThermoFisher Scientific).

Fragment fluorescence was quantified using Adobe Photoshop to obtain integrated density of each band. Instances where the intensity of competitor band and either target band appeared equal were classified as a 'zone of equivalence' for the DNA of each parasite. Where a zone of equivalence was intermediate between 2 adjacent lanes, the concentration was estimated mathematically by interpolation from the 2 known values bounding the zone of equivalence to determine 'competition equivalence point' for DNA level of each parasite.

2.5. Analysis

Estimated concentrations of *H. nelsoni* and *P. marinus* DNA amplified from water samples were normalized for filtration and dilution volumes. Data were transformed using a Box-Cox transformation. Dunn's tests were performed among the 3 groups independently for both infectious agents to determine if there was an overall difference among sites for detected quantities of *H. nelsoni* and *P. marinus*. Time series regressions were performed for each parasite separately with temperature and over time. All analyses were performed in R (v 4.3.3, R Core Team 2020).

3. RESULTS

The collection, preservation, and extraction methods yielded approximately 100 μl of extracted DNA at a concentration range of 5.1–13.2 ng μl^{-1} free of PCR inhibitors. At least 1 of the 2 pathogens was detected in every water sample (n = 78), but there were no clear trends in presence or absence of either parasite DNA, neither spatially (both $p > 0.5$) nor temporally (both $p > 0.1$). Levels of *Haplosporidium nelsoni* DNA in GBE exhibited a seasonal pattern, rising throughout July to an August peak and then decreasing throughout September to low levels. Very high levels of *H. nelsoni* (>0.1 pg m^{-3}) were found from late July through late August. During the summer, the level of *H. nelsoni* DNA was significantly higher at the reef site than at the farm site ($p = 0.001$), and both reef and farm were significantly higher than the reference site ($p < 0.001$ for both locations) (Fig. 2A). The highest concentration of *H. nelsoni* DNA was found on 19 August at the reef (Fig. 3). At the inception of

Table 1. Primers (W. T. Brooks unpubl. data) used for PCR and quantitative competitive PCR (QCPCR)

Primer	Sequence
HnPm-A	5'-AGC CAT GCA TGT CTA AGT ATA A-3'
HnePsp-B	5'-GAT GTG GTA GCC GTT TCT CAG G-3'
HnePsp-BC	5'-GAT GTG GTA GCC GTT TCT CAG GGC CCA TAT CCT ACC GTC AAG C-3'

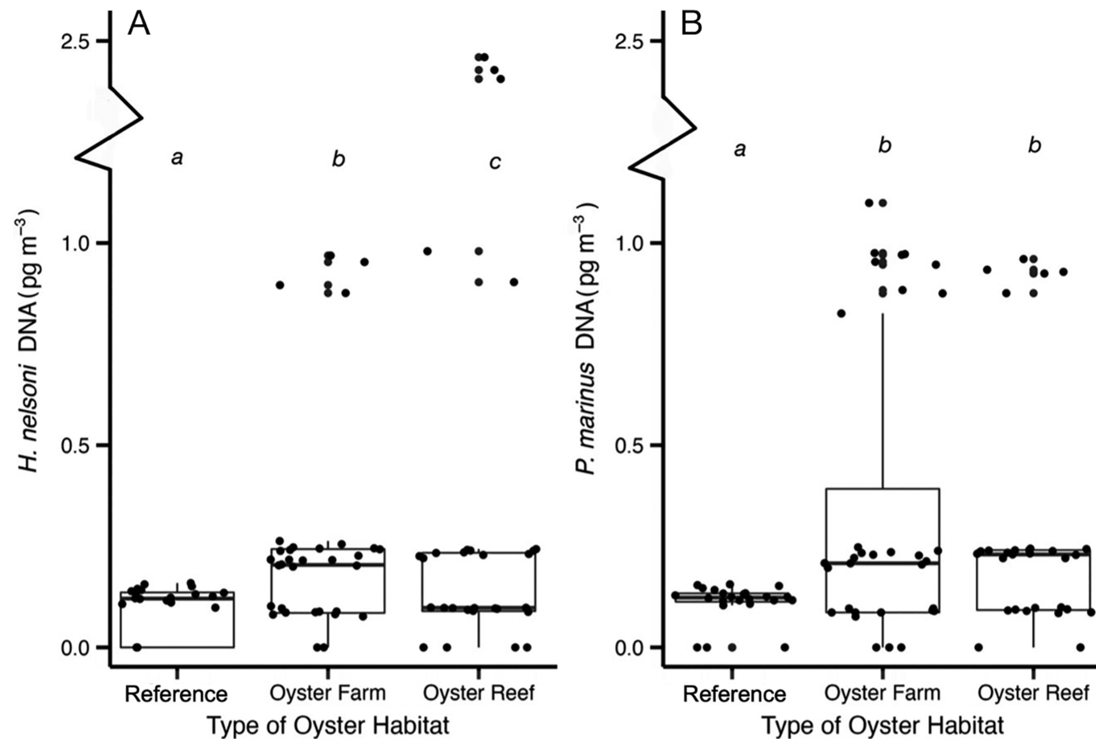

Fig. 2. Comparison of (A) *Haplosporidium nelsoni* and (B) *Perkinsus marinus* DNA levels in waters at an oyster farm, oyster reef, and a non-oyster reference site in Great Bay Estuary, New Hampshire. Sites with the same lowercase letter are not significantly different from one another; those with different lowercase letters are significantly different at $p \leq 0.01$. Box: inter-quartile range (IQR); bold solid line: mean; whiskers: 1.5 × IQR; black dots: DNA levels of *H. nelsoni* and *P. marinus*

the study in late spring, *Perkinsus marinus* DNA already was quite high at both oyster sites and differed significantly among the 3 sites ($p < 0.001$). The oyster sites maintained levels of *P. marinus* DNA that were significantly higher than at the reference site ($p < 0.001$) throughout the period of examination (Fig. 2B). The highest concentration for *P. marinus* was found on 9 June at the farm site.

Although not an overall goal of this project, post hoc PCR tests were performed on 10 individual oyster larvae (D-hinge), 2 unidentified bivalve larvae, and 2 crab zoeae. Four of the oyster larvae yielded positive PCR results for *H. nelsoni*, and none of the other plankters tested positive for either parasite.

4. DISCUSSION

Both MSX and dermo have caused catastrophic damage to eastern oyster populations along the eastern US Atlantic coast. *Haplosporidium nelsoni* abundance is influenced by complex relationships among salinity, temperature, parasite burden, and other factors (Burreson & Ford 2004). The parasite has optimal infectivity at high salinities, and GBE salinities are consistently >20 ppt (Stasse et al. 2022). The mode by which *H. nelsoni* infects oysters, while studied extensively, still is not fully understood (Ford et al. 2018). Potential intermediate hosts have been tested (Messerman & Bowden 2016), as well as direct transmission among oysters, and these have not been found to be successful mechanisms of transmission. Determining the sources of DNA that were detected in this study (i.e. were there spores in the water, were oyster larvae infected, was there another organism carrying the pathogen, etc.) is a route for further research that could help identify the means for infection transmission.

Perkinsus marinus requires temperatures above 20°C and salinities from 12–15% to proliferate in eastern oysters, but it can survive throughout the winter at temperatures as low as 0–5°C and at salinities <5% (Andrews & Hewatt 1957). These conditions occur in GBE, providing the opportunity for *P. marinus* to persist. Contrary to *H. nelsoni*, the transmission of *P. marinus* is known; trophozoites from dead, infected oysters enter oyster haemocytes (Tasumi & Vasta 2007), reproduce, and dermo disease eventually leads to mortality. Once the oyster dies from infection, trophozoites released into the water cause subsequent infection in other oysters (Alavi et al. 2009). The ob-

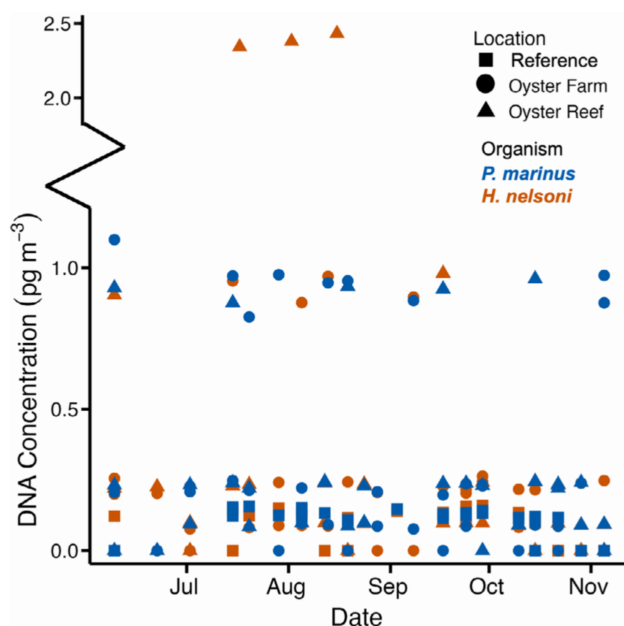

Fig. 3. Trends in water concentration of *H. nelsoni* and *P. marinus* DNA in Great Bay Estuary, New Hampshire

served trend in GBE water levels of *P. marinus* and the high levels in June that coincided that year with a week of high temperatures may be a signal of an oyster die-off that could have led to increased infection in July and August. Since zoospores are released into the water after oysters die, the increased *P. marinus* levels observed in November may be related to high oyster mortality in the autumn season.

The importance of water monitoring to examine transmission dynamics of these 2 parasites has been highlighted (Audemard et al. 2006, Messerman & Bowden 2016, Gignoux-Wolfsohn et al. 2021). Investigators have queried individual plankters for *H. nelsoni* (Ford et al. 2018) and did not detect a non-oyster host. Investigators have detected free-living stages of *P. marinus* in environmental waters using methodologies that include centrifugation, histology, immunochemistry, and RT-PCR (Ragone Calvo et al. 2003, Audemard et al. 2004, Ellin & Bushek 2006, Dungan et al. 2012). The present study introduces a single QCPCR assay to detect and quantify DNAs of *H. nelsoni* and *P. marinus* simultaneously. Significantly higher concentrations of *H. nelsoni* DNA were found in late season at both oyster reef ($p = 0.001$) (maximum = 2.5 pg m^{-3}) and oyster farm water samples (maximum = 0.9 pg m^{-3}). Noting that there was a much higher density of oysters at the farm than on the reef, the lower levels of parasite DNA in farmed oyster water are most likely due to predominance at the farm of oysters selectively bred for MSX-resistance versus wild oysters on the reef. Our observation of no clear temporal progression in DNA quantity is similar to observations of *P. marinus* cells in the water column (Ragone Calvo et al. 2003), i.e. early and late season 'spikes' as opposed to a progression of interval average parasite abundance as observed in James River, a tributary of Chesapeake Bay (Audemard et al. 2006). Inspection of Fig. 3 may appear to illustrate 'steps' of parasite DNA. This is an artifact of the logarithmic-scale competitor dilutions used in this study, where interpolated DNA concentrations are clustered around the competitor points (Fig. 3). Future studies using this technique should include competitor dilutions on a linear scale (e.g. 0, 0.5, 1.0, 2.0, and 3.0 pg µl^{-1}).

QCPCR is a method accessible to even the most modestly outfitted laboratory and, with simple post-PCR manipulation, could be employed in diverse systems to survey for DNA from pathogens and determine whether they are individual cells or incorporated in other plankters. Even so, determining species abundance from DNA concentration is extremely challenging as there is a complex relationship between the two (Jo & Yamanaka 2022). The trends observed in abundance of *H. nelsoni* and *P. marinus* throughout GBE waters offer several avenues for further research and aid in environmental monitoring. Presence of *H. nelsoni* DNA in the water suggests that either *H. nelsoni* occurs as infective particles in the water or is within another planktonic organism. The post hoc PCR test of oyster, bivalve, and crab larvae collected from GBE water samples adds to what is learned from the prior study of plankters to determine their ability to act as an intermediate host for *H. nelsoni* (Ford et al. 2018). The revelation that 4 of 10 individual oyster larvae tested were positive for *H. nelsoni* DNA indicates that oyster larvae could be a potential venue by which MSX disease spreads, and this warrants a thorough study.

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LITERATURE CITED

Alavi MR, Fernández-Robledo JA, Vasta GR (2009) Development of an in vitro assay to examine intracellular survival of *Perkinsus marinus* trophozoites upon phagocytosis by oyster (*Crassostrea virginica* and *Crassostrea ariakensis*) hemocytes. J Parasitol 95:900–907

Andrews JD, Hewatt WG (1957) Oyster mortality studies in Virginia. II. The fungus disease caused by *Dermocystidium marinum* in oysters of Chesapeake Bay. Ecol Monogr 27:1–25

Audemard C, Reece KS, Burreson EM (2004) Real-time PCR for detection and quantification of the protistan parasite *Perkinsus marinus* in environmental waters. Appl Environ Microbiol 70:6611–6618

Audemard C, Ragone Calvo LM, Paynter KT, Reece KS, Burreson EM (2006) Real-time PCR investigation of parasite ecology: *in situ* determination of oyster parasite *Perkinsus marinus* transmission dynamics in lower Chesapeake Bay. Parasitology 132:827–842

Barber BJ, Langan R, Howell TL (1997) *Haplosporidium nelsoni* (MSX) epizootic in the Piscataqua River Estuary (Maine/New Hampshire, U.S.A.). J Parasitol 83:148–150

Beck M, Brumbaugh R, Airoldi L, Carranza A and others (2011) Oyster reefs at risk and recommendations for conservation, restoration, and management. Bioscience 61:107–116

Brandon CM, Woodruff JD, Orton PM, Donnelly JP (2016) Evidence for elevated coastal vulnerability following large-scale historical oyster bed harvesting. Earth Surf Process Landf 41:1136–1143

Burreson EM, Ford SE (2004) A review of recent information on the *Haplosporidia*, with special reference to *Haplosporidium nelsoni* (MSX disease). Aquat Living Resour 17:499–517

Day JM, Franklin DE, Brown BL (2000) Use of competitive PCR to detect and quantify *Haplosporidium nelsoni* infection (MSX disease) in the eastern oyster (*Crassostrea virginica*). Mar Biotechnol 2:456–465

Dungan CF, Carnegie RB, Hill KM, McCollough CB and others (2012) Diseases of oysters *Crassostrea ariakensis* and *C. virginica* reared in ambient waters from the Choptank River, Maryland and the Indian River Lagoon, Florida. Dis Aquat Org 101:173–183

Eckert RL (2016) Oyster (*Crassostrea virginica*) recruitment studies in the Great Bay estuary, New Hampshire. MSc thesis, University of New Hampshire, Durham, NH

Ellin R, Bushek D (2006) Adaptation of Ray's fluid thioglycollate medium assay to detect and quantify planktonic stages of *Perkinsus* spp. parasites. J Shellfish Res 25:1037–1042

Ford SE, Haskin HH (1987) Infection and mortality patterns in strains of oysters *Crassostrea virginica* selected for resistance to the parasite *Haplosporidium nelsoni* (MSX). J Parasitol 73:368–376

Ford SE, Stokes NA, Alcox KA, Kraus BSF, Barber RD, Carnegie RB, Burreson EM (2018) Investigating the life cycle of *Haplosporidium nelsoni* (Msx): a review. J Shellfish Res 37:679–693

Gignoux-Wolfsohn SA, Newcomb MSR, Ruiz GM, Pagenkopp Lohan KM (2021) Environmental factors drive the release of *Perkinsus marinus* from infected oysters. Parasitology 148:532–538

Grizzle R, Ward K, Konisky R, Greene J, Abeels H, Atwood R (2021) Oyster reef restoration in New Hampshire, USA: lessons learned during two decades of practice. Ecol Restor 39:260–273

Guo X (2021) Genetics in shellfish culture. In: Shumway E (ed) Molluscan shellfish aquaculture: a practical guide. 5M Books, Great Easton, p 393–413

Haney JF, Hall DJ (1973) Sugar-coated *Daphnia*: a preservation technique for Cladocera. Limnol Oceanogr 18:331–333

Haskin HH, Ford SE (1979) Development of resistance to *Minchinia nelsoni* (MSX) mortality in laboratory-reared and native oyster stocks in Delaware Bay. Mar Fish Rev 42:54–63

Jo T, Yamanaka H (2022) Fine-tuning the performance of abundance estimation based on environmental DNA (eDNA) focusing on eDNA particle size and marker length. Ecol Evol 12:e9234

Messerman NA, Bowden TJ (2016) Survey of potential reservoir species for the oyster parasite multinucleate sphere X (*Haplosporidium nelsoni*) in and around oyster farms in the Damariscotta River Estuary, Maine. J Shellfish Res 35:851–856

Newell RIE, Fisher TR, Holyoke RR, Cornwell JC (2005) Influence of eastern oysters on nitrogen and phosphorus regeneration in Chesapeake Bay, USA. In: Dame RF, Olenin S (eds) The comparative roles of suspension-feeders in ecosystems. NATO Science Series IV: Earth and Environmental Series. Springer, Dordrecht, p 93–120

Patterson CA, Sullivan KM (2020) Testing of Great Bay oysters for two protozoan pathogens. PREP (Piscataqua Region Estuaries Partnership) Rep Publ 440. University of New Hampshire, Durham, NH

R Core Team (2020) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Ragone Calvo LM, Dungan CF, Roberson BS, Burreson EM (2003) Systematic evaluation of factors controlling *Perkinsus marinus* transmission dynamics in lower Chesapeake Bay. Dis Aquat Org 56:75–86

Stasse A, Cheng MLH, Meyer K, Bumbera N and others (2022) Temporal dynamics of eastern oyster larval abundance in Great Bay Estuary, New Hampshire. J Shellfish Res 40:471–478

Tasumi S, Vasta GR (2007) A galectin of unique domain organization from hemocytes of the eastern oyster (*Crassostrea virginica*) is a receptor for the protistan parasite *Perkinsus marinus.* J Immunol 179:3086–3098

Yarnall HA, Reece KS, Stokes NA, Burreson EM (2000) A quantitative competitive polymerase chain reaction assay for the oyster pathogen *Perkinsus marinus.* J Parasitol 86:827–837

Zentilin L, Giacca M (2010) The renaissance of competitive PCR as an accurate tool for precise nucleic acid quantification. Methods Mol Biol 630:233–248

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