2022

Development of a multiplex qPCR for the quantification of three protozoan parasites of the eastern oyster Crassostrea virginica

Jessica L. Piesz
University of Rhode Island

Abigail K. Scro

Ryan Corbett
University of Rhode Island

Kathryn Markey Lundgren

Roxanna Smolowitz

See next page for additional authors

Citation/Publisher Attribution

Piesz JL, Scro AK, Corbett R, Markey Lundgren K, Smolowitz R, Gomez-Chiarri M (2022) Development of a multiplex qPCR for the quantification of three protozoan parasites of the eastern oyster *Crassostrea virginica*. Dis Aquat Org 151:111-121. https://doi.org/10.3354/dao03694
Available at: https://doi.org/10.3354/dao03694

Vol. 151: 111–121, 2022
https://doi.org/10.3354/dao03694

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published online October 27

Development of a multiplex qPCR for the quantification of three protozoan parasites of the eastern oyster *Crassostrea virginica*

Jessica L. Piesz[1], Abigail K. Scro[2], Ryan Corbett[1], Kathryn Markey Lundgren[2,3], Roxanna Smolowitz[2], Marta Gomez-Chiarri[1,*]

[1]Department of Fisheries, Animal, and Veterinary Science, University of Rhode Island, Kingston, Rhode Island 02881, USA
[2]Aquatic Diagnostic Laboratory, Roger Williams University, Bristol, Rhode Island 02809, USA
[3]National Cold Water Marine Aquaculture Center, USDA, CBLS 469, Kingston, Rhode Island 02881, USA

ABSTRACT: A multiplex quantitative PCR (qPCR) assay for the simultaneous detection of 3 eastern oyster *Crassostrea virginica* parasites, *Perkinsus marinus*, *Haplosporidium nelsoni*, and *H. costale*, was developed using 3 different fluorescently labeled hydrolysis probes. The primers and probe from a previously validated singleplex qPCR for *P. marinus* detection were combined with newly designed primers and probes specific for *H. nelsoni* and *H. costale*. The functionality of the multiplex assay was demonstrated on 2 different platforms by the linear relationship of the standard curves and similar cycle threshold (C_T) values between parasites. Efficiency of the multiplex qPCR assay on the Roche and BioRad platforms ranged between 93 and 101%. The sensitivity of detection ranged between 10 and 100 copies of plasmid DNA for *P. marinus* and *Haplosporidium* spp., respectively. The concordance between the Roche and BioRad platforms in the identification of the parasites *P. marinus*, *H. nelsoni*, and *H. costale* was 91, 97, and 97%, respectively, with a 10-fold increase in the sensitivity of detection of *Haplosporidium* spp. on the BioRad thermocycler. The concordance between multiplex qPCR and histology for *P. marinus*, *H. nelsoni*, and *H. costale* was 54, 57, and 87%, respectively. Discordances between detection methods were largely related to localized or low levels of infections in oyster tissues, and qPCR was the more sensitive diagnostic. The multiplex qPCR developed here is a sensitive diagnostic tool for the quantification and surveillance of single and mixed infections in the eastern oyster.

KEY WORDS: *Crassostrea virginica* · Eastern oyster · Diagnostic assay · *Perkinsus marinus* · *Haplosporidium nelsoni* · *Haplosporidium costale* · qPCR

1. INTRODUCTION

The protozoan parasites *Perkinsus marinus*, *Haplosporidium nelsoni*, and *H. costale*, the causative agents of dermo disease, multinucleate sphere unknown (MSX), and seaside organism (SSO), respectively, are a significant cause of mortalities of wild and farmed eastern oysters *Crassostrea virginica* along the Gulf of Mexico and east coast of the USA (Burreson & Ford 2004, Villalba et al. 2004, Soudant et al. 2013). Recently, an outbreak of SSO was also reported in Pacific oysters *Crassostrea gigas* in France (ICES 2020). Low levels of these parasites in oysters can occur with no obvious clinical impact, but at higher infections, oysters become thin and watery as tissue and shell growth is slowed, eventually leading to death (Ray et al. 1953, Menzel & Hopkins 1955, Barber et al. 1988).

These protozoan parasites are believed to enter *C. virginica* through the filter feeding processes of the

*Corresponding author: gomezchi@uri.edu

Publisher: Inter-Research · www.int-res.com

oyster, commonly first infecting the gill, mantle tissues, and digestive gland (depending on the parasite). *H. nelsoni* proliferates in the gill epithelium before breaking through the basement membranes underlying the gill epithelium and then entering the circulatory system (Myhre & Haskin 1967, Farley 1968, Ford 2002). In advanced infection cases, parasite cells may be easily observed in the vascular system adjacent to the digestive gland as well as the gills (Perkins 1969, Andrews & Castagna 1978). Alternatively, infectious *H. costale* are primarily found surrounding the digestive system of *C. virginica* as spores are ingested. The spores invade through the gastric and digestive gland epithelium, become multinucleated (similar to MSX), and then disseminate throughout the vascular system of the oyster, but in early cases are most often found histologically adjacent to the digestive system. Both *H. nelsoni* and *H. costale* are similar in appearance, making identification difficult through histological examination (Perkins 1969, Ford & Tripp 1996). Traditionally, a combination of rectum, mantle, and gill tissue samples is used for the detection of *P. marinus* using Ray's fluid thioglycollate medium (RFTM) culture assay (Ray 1952), based on the higher parasite abundance in these tissues. Emergence of a new *P. marinus* phenotype in the 1980s, showing a shift in the presence of the parasite from connective to epithelial tissues, may have allowed for disease expansion through increased transmission via feces (Carnegie et al. 2021).

Prevalence and intensity of these 3 infections in the natural environment vary by region and are heavily influenced by environmental conditions. Increasing temperature (>20°C) and salinity (>15 PSU) has been shown to increase parasite load and the prevalence of infection in the environment (Ford & Tripp 1996, Burreson & Ford 2004, Villalba et al. 2004). Disease-associated mortalities are responsible for large economic losses to the aquaculture industry and to wild oysters, as epizootic events have been recorded periodically along the US Atlantic coast (Andrews & Hewatt 1957, Haskin et al. 1966, Barber et al. 1997, Sunila et al. 1999). Failure to monitor disease levels could result in future mortality events, especially with the transplant of healthy or naïve individuals into areas where these parasites are present (Ford & Haskin 1987, Ragone Calvo et al. 2003). With the expansion of oyster aquaculture and continuation of restoration efforts, there is a greater need for monitoring programs that offer economic, accurate, and quick diagnoses of these parasites. Shellfish-producing states in the USA now require disease testing as part of their Shellfish Strategic Plan, and some, such as Rhode Island, set up biosecurity zones based on historical and/or current parasite levels in various bodies of water (CRMC 2012). The World Organisation for Animal Health (OIE) lists *P. marinus* as a reportable disease (Carnegie et al. 2016, OIE 2021).

Monitoring programs utilize a variety of diagnostic techniques to assess the health and condition of farmed and wild oysters. Conventional diagnostic tools such as histology and RFTM allow for the visualization or the culture of the parasite in infected tissues, while *in situ* hybridization is a specialized technique used to verify a parasite in a tissue section. All of these techniques require trained experts for accurate diagnosis and can be costly and time consuming, and some (e.g. *in situ* hybridization) are not practical as a standard diagnostic technique (Ray 1952, Wood & Andrews 1962, Andrews & Castagna 1978). PCR provides a faster and more sensitive alternative to conventional diagnostic methods, and has been extensively used in the diagnosis of these parasites (Stokes et al. 1995, Robledo et al. 1998, Yarnall et al. 2000, Stokes & Burreson 2001). However, gel-based PCR does not quantify the abundance of parasites in the tissues, and thus, it does not replace the information provided by conventional methods such as histology and RFTM. Improved PCR assays such as real-time or quantitative PCR (qPCR) have replaced gel-based PCR methods (those in which final products from amplification are visualized through gel electrophoresis) by providing a more sensitive and quantitative measurement of pathogen presence in oysters (Audemard et al. 2004, De Faveri et al. 2009, Wilbur et al. 2012, Xie et al. 2013, López-Sanmartín et al. 2019, Arzul et al. 2022). Furthermore, qPCR assays can be multiplexed to allow for more economic and faster diagnostics. Multiplexing allows for the simultaneous detection of multiple targets in a single reaction through the use of multiple fluorescent dyes with non-overlapping wavelengths that will recognize and differentiate between multiple targets (i.e. species of parasites in this case).

The aim of this paper was to develop an efficient and sensitive TaqMan® multiplex qPCR assay for the high-throughput detection and quantification of 3 economically important oyster parasites. Oyster parasite detection by multiplex qPCR was validated at the University of Rhode Island (Kingston, RI) on a Roche thermocycler and at Roger Williams University (Bristol, RI) on a BioRad thermocycler, and performance was compared against traditional techniques (e.g. histology, singleplex qPCR, and gel-based PCR) to confirm assay functionality. This assay will reduce the

cost of analysis by simplifying detection formats, thus allowing for the sensitive detection of multiple parasites in a single reaction. The ease of use and speed of the assay will allow for a more rapid routine diagnosis and a quicker corrective response, helping minimize production losses to the aquaculture industry.

2. MATERIALS AND METHODS

2.1. Sampling and DNA extraction

Live oysters or previously extracted genomic DNA from infected oysters were donated from multiple locations along the east coast of the USA (n = 130; Table 1). Extractions were performed at the University of Rhode Island using a single or a high-throughput Chelex DNA extraction protocol adapted from Aranishi & Okimoto (2006). Sections of preserved (in 95% ethanol) mantle and gill tissue were weighed and 5.0 mg were added to individual 1.5 ml microcentrifuge tubes or the wells of a 96-well deep-well plate containing 100 or 200 µl of urea buffer, respectively. Buffer was composed of 4 M urea, 1% Tween 20, 1% Triton X-100, 5% Chelex 100, and 5 µg Proteinase K in deionized water. The samples were vortexed for 30 s and heated in an incubator (1.5 ml tubes) or thermocycler (96-well plate) to 55°C for 60 min and then at 100°C for 8 min. For the high throughput protocol, plates were vortexed at 30 and 60 min followed by centrifugation at 3500 × *g* for 20 min. For single extractions, 1.5 ml tubes were centrifuged for 5 min at 15 000 × *g*. Aliquots (100 µl) of the supernatant were collected from each extraction and added to a clean 1.5 ml tube or deep-well plate containing 1 µl 100TE buffer (pH 8.0) (1 M Tris-HCl [pH 8.0], 100 mM EDTA [pH 8.0]), 50 µl 7.5 M ammonium acetate, and 400 µl 100% ethanol to precipitate the DNA. Samples were vortexed for 30 s and centrifuged for 20 min at 15 000 × *g* (single extractions) or 3500 × *g* (96-well) to pellet the DNA. Ice-cold 70% ethanol (200 µl) was added to each well, vortexed gently, and centrifuged for 5 min at 15 000 × *g* (single extractions) or 20 min at 3500 × *g* (96-well). Pellets were washed 2 more times, air dried, and resuspended in 50 or 100 µl of 100TE buffer. The concentration of DNA was quantified using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific™) and diluted to 50 ng µl^{-1} in nuclease-free water. Samples were stored at −20°C until further use.

Table 1. Oyster samples used in the validation of the multiplex qPCR assay. Samples (n = 130) were collected along the Atlantic coast of the USA

Sources of samples	n
Cape Fear River estuary, North Carolina	5
Delaware Bay, New Jersey	22
Long Island Sound, Connecticut	13
Pawcatuck River, Rhode Island	4
Point Judith Pond, Rhode Island	2
Mount Hope Bay, Rhode Island	6
Wellfleet, Massachusetts	4
Kennebec River, Maine	5
Damariscotta River, Maine	2
Bagaduce River, Maine	1
Pemaquid River, Maine	66

2.2. Primer and probe design

The complete sequence and partial sequences of the small subunit ribosomal RNA (SSU rRNA) genes for *Haplosporidium costale* (accession numbers U20858, AF387122; Ko et al. 1995, Stokes & Burreson 2001) and *H. nelsoni* (AB080597, U19538, X74131; Stokes et al. 1995), as well as 47 other conserved sequences (e.g. *Minchinia*, *Bonamia*, and *Haplosporidium* spp.) were downloaded from GenBank and aligned with MUSCLE to identify gene-specific regions for primer and probe design (Edgar 2004). Using IDT's OligoAnalyzer™ tool, a single pair of conserved primers was designed to amplify the same region for both haplosporidian species, while TaqMan® probes specific to *H. nelsoni* and *H. costale* were designed to differentiate between these 2 parasites (Table 2). For *Perkinsus marinus* detection, we used previously validated primer and probe sequences from De Faveri et al. (2009). The probe and primer sequences were routinely checked for specificity based on matches with sequences in non-redundant genetic databases using BLAST (last search performed on 28 March 2022).

2.3. Multiplex qPCR reaction

The multiplex assay was first optimized on the LightCycler® 480 (Roche) qPCR machine at the University of Rhode Island. Reaction mix components included 450 nM of the *Haplosporidium* spp. primers, 300 nM of the *P. marinus* primers, 75 nM of each species-specific probe, 10 µl of LightCycler® 480 probes master mix, and 2 µl of 50 ng µl^{-1} DNA template. Distilled water was used to bring the final reaction volume to 20 µl. The thermal cycling protocol consisted of a 95°C hold for 10 min followed by 45 cycles of

Table 2. Oligonucleotide primers and probes used in multiplex qPCR assay. IAC: internal amplification control

Target	Direction	Sequence (5' → 3')	Reference
Perkinsus marinus	Forward	CGC CTG TGA GTA TCT CTC GA	De Faveri et al. (2009)
	Reverse	GTT GAA GAG AAG AAT CGC GTG AT	
	Probe	5'6-FAM/CGC AAA CTC GAC TGT GTT GTG GTG/3'BHQ _1	
Haplosporidium sp.	Forward	ACA GGT CAG TGA TGC CCT TAG	This study
Haplosporidium sp.	Reverse	TSG RGA TTA CCY SGC CTT C	
H. costale	Probe	5'Cy5/AAT GAC CCA GTC AGC GGG CCG A/3'BHQ_2	
H. nelsoni	Probe	5'HEX/TTG CAC GCA ACG AGT TCA ACC TTG CCT G/3'BHQ_1	
IAC	Forward	GAC ATC GAT ATG GGT GCC G	Nordstrom et al. (2007)
	Reverse	CGA GAC GAT GCA GCC ATT C	
	Probe	5'Cy5/TCT CAT GCG TCT CCC TGG TGA ATG TGA ATG TG/3'BHQ_2	

amplification (95°C for 15 s, 57°C for 1 min, and 72°C for 1 s). Controls included deionized water, spiked positive oyster genomic DNA (see Section 2.4), and genomic DNA of naïve oysters from the Gulf of Mexico where MSX and SSO have not been detected in widespread disease surveys (Ford et al. 2011, OIE 2021).

2.4. Preparation of standards and spiked positive controls

Cloned targets for *H. costale*, *H. nelsoni*, and *P. marinus* were prepared from genomic DNA from Rhode Island infected oysters. Each target was amplified using species-specific primers to the SSU rRNA gene (Table 3) and cloned into the pCR™II-TOPO® or pCR®4-TOPO® vector using an Invitrogen TOPO TA Cloning Kit. Plasmid DNA was isolated from positive clones using a Qiagen Plasmid Mini Kit and sequenced to confirm gene insert. Ten-fold dilutions (10^7–10^1 copies) of each target were prepared as standards for each qPCR reaction. A high and low positive oyster spiked control was prepared by spiking pooled genomic DNA from 13 parasite-free oysters (as determined by gel-based PCR and histological examination) with 10^5 and 10^2 copies of plasmid DNA, respectively, from each target.

2.5. Internal amplification control (IAC)

Biological samples may contain substances that degrade nucleic acids or inhibit the polymerase activity of a PCR reaction (Hoorfar et al. 2004, Espy et al. 2006, Rodríguez et al. 2012). To screen for PCR inhibition by the sample matrix in this assay, an IAC from Nordstrom et al. (2007) (Table 2) was tested in 2 separate reactions with *P. marinus* and *H. nelsoni*. Due to the identical use of fluorophore for the IAC probe, *H. costale* was omitted. Samples included infected and parasite-free oyster genomic DNA extracted using the Chelex protocol described in Section 2.1 (n = 26). The IAC was designed to give a cycle threshold (C_T) value of 20 ± 1 under non-inhibitory conditions regardless of the presence of parasites in the reaction mixture (Nordstrom et al. 2007).

2.6. Sensitivity and specificity of qPCR multiplex

The sensitivity of the assay at diagnosing *P. marinus*, *H. nelsoni*, and *H. costale* infections was evaluated by comparing the multiplex qPCR C_T values for each sample to other diagnostic methods such as histology, gel-based PCR (Stokes & Burreson 2001), or singleplex qPCR (De Faveri et al. 2009, Wilbur et

Table 3. Primers used for cloning of *Haplosporidium nelsoni*, *H. costale*, and *Perkinsus marinus* gene-specific sequences

Target	Primer sequence	Size (bp)	Amplified region (gene)	Reference
H. nelsoni	5'-TGG CAT TAG GTT TCA GAC CT-3'	725	SSU rRNA	Russell et al. (2004)
	5'-TSG RGA TTA CCY SGC CTT C-3'			This study
H. costale	5'-CAC GAC TTT GGC AGT TAG TTT TG-3'	718	SSU rRNA	Stokes & Burreson (2001)
	5'-TSG RGA TTA CCY SGC CTT C-3'			This study
P. marinus	5'-CGC CTG TGA GTA TCT CTC GA-3'	90	SSU rRNA	De Faveri et. al. (2009)
	5'-GTT GAA GAG AAG AAT CGC GTG AT-3'			

al. 2012). Analytical sensitivity was determined by running 10-fold dilutions (10^7–10^1 copies) of plasmid DNA for each target. The lower limit of quantification was defined as the lowest copy number that can be accurately measured by multiplex qPCR (i.e. C_T values on the linear regression line). The efficiency (E) of the amplification of each target was calculated using the slope (S) of the linear regression line ($E = 10^{(-1/S)} - 1$) of 10-fold dilutions of plasmid standards for each target. In order to determine the specificity of the primer sets (no probe present), a separate dissociation curve analysis (i.e. melting curve analysis) was performed using the SYBR Green qPCR assay on the Roche platform following previously described protocols (Roche Life Technologies 2012).

2.7. Reproducibility

A subsample of each extracted oyster tissue sample (n = 34) was tested in duplicate by multiplex qPCR at 2 different laboratories and platforms, namely a LightCycler® 480 thermocycler (Roche) at the University of Rhode Island and a CFX96C1000 thermocycler (BioRad) at Roger Williams University, to evaluate the reproducibility of the assay. Intra-assay variation was assessed in samples by calculating the average of the coefficient of variation (CV) of the replicate wells within a single assay ($CV = \sigma/\mu \times 100$; where σ and μ are the SD and mean of the data set, respectively). Inter-assay variation between qPCR platforms was evaluated by calculating the average of the CVs for an individual sample tested on each platform. Variations ≤5% were considered acceptable.

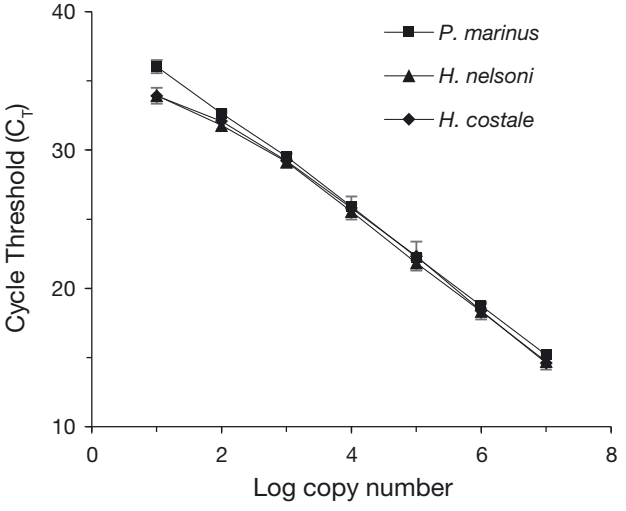

Fig. 1. Standard curves for the amplification of *Perkinsus marinus*, *Haplosporidium nelsoni*, and *H. costale* targets. The efficiency (E) of the qPCR multiplex was evaluated by running 10-fold dilutions (10^7–10^1 copies) of plasmid DNA for each target on the Roche Lightcycler® 480 platform. Data show the mean ± SD of 4 individual qPCR assays. *P. marinus* (E = 93.6%); *H. nelsoni* (E = 101.7%); *H. costale* (E = 101.1%)

2.8. Histological analysis

Diagnosis was also performed by histology using a subset of 121 of the 130 samples. Each oyster was shucked and fixed in 10% formalin in seawater. Standard cross sections were removed and processed in paraffin using standard methods (Ford & Tripp 1996, Howard et al. 2004). Paraffin slices were cut to 6 µm, mounted on glass slides, and stained using hematoxylin and eosin (H&E). Histological sections were analyzed using a compound microscope (model varied by laboratory).

3. RESULTS

3.1. Specificity and sensitivity of qPCR multiplex

Blast analysis of the *Haplospodium costale* and *H. nelsoni* probe sequences showed that these newly designed probes were specific for each of the respective target species and did not fully match the sequence for any other haplosporidian or marine protist (not shown). The sensitivity of the multiplex qPCR assay was demonstrated by the broad linear range of detection of 10-fold dilutions (10^7–10^1 copies) of plasmid DNA for each target (Fig. 1). The sensitivity of detection was as low as 10 and 100 copies for *Perkinsus marinus* and *Haplosporidium* spp., respectively, using the Roche LightCycler® 480 platform. The reduced sensitivity of the assay in detecting *H. nelsoni* and *H. costale* was reflected in the standard curves by the non-linear amplification between 10 and 100 copies of plasmid DNA (Fig. 1). Dissociation curve analysis of *Haplosporidium* spp. standards revealed primer-dimer formation at low template concentrations (10^1 and 10^2 copies; not shown). The non-specific amplification was determined to be the explanation for the reduced sensitivity of the assay in detecting low target concentrations. For this reason, the lower limit of quantification of these targets was set at 10 copies (for *P. marinus*, C_T = 36) and 100 copies (for *H. nelsoni* and *H. costale;* C_T = 31) for the Roche platform. The standard curves from 4 replicate multiplex qPCR assays showed overlapping slopes for each individual target, which suggested that the reaction conditions were optimal (Fig. 1).

Table 4. Oyster parasite multiplex qPCR diagnostic specificity. Multiplex qPCR diagnostic specificity evaluation against singleplex qPCR (for *Perkinsus marinus*, De Faveri et al. 2009; and *Haplosporidium nelsoni*, Wilbur et al. 2012) and gel-based PCR (for *H. costale,* Stokes & Burreson 2001). Rate indicates the number of samples in which both methods agreed/total number of samples tested. Concordance between detection methods and qPCR platforms ranged from 80 to 100%

Multiplex qPCR	Singleplex qPCR				Gel-based qPCR	
	P. marinus		*H. nelsoni*		*H. costale*	
	Rate	%	Rate	%	Rate	%
Roche	5/5	100	8/9	89	6/6	100
BioRad	4/5	80	8/9	89	6/6	100

The percent concordance between multiplex qPCR, singleplex qPCR for *P. marinus* and *H. nelsoni*, and the gel-based PCR assay for *H. costale* detection was 80–100% (Table 4). The same primers and probe set were used in the singleplex and multiplex assays for *P. marinus*. This is not true for the gel-based PCR assay for SSO, in which a larger amplicon is targeted (Stokes & Burreson 2001), nor the singleplex assay for *H. nelsoni* (different amplicons were targeted in the multiplex assay as compared to the singleplex developed by Wilbur et al. 2012). There were 2 discordant samples between PCR detection formats. One of these samples was positive for *H. nelsoni* by singleplex qPCR (C_T = 37) but negative by multiplex qPCR on both Roche and BioRad platforms. The remaining discordant sample was negative by multiplex qPCR on the BioRad platform but was positive for *P. marinus* by singleplex qPCR (C_T = 18).

PCR inhibition by the sample matrix (oyster tissue) was not detected in oyster samples extracted using Chelex (n = 26), with all oyster samples showing C_T values within the established limits of the IAC control (21 ± 1) (data not shown).

3.2. Reproducibility

The performance of the multiplex assay at detecting *P. marinus* and *Haplosporidium* spp. developed using the Roche platform was reproducible on the BioRad thermocycler with amplification efficiencies ranging from 91 to 94% (Table 5). Standard curves showed a wide range of detection, 10^7–10^1 copies of plasmid DNA for all targets and in the BioRad platform and for *Perkinsus marinus* in the Roche platform, and 10^7–10^2 for *H. nelsoni* and *H. costale* infections on the Roche platform (Figs. 1 & 2). Assay sensitivity and specificity were evaluated between platforms by comparing the C_T values of 34 samples of oyster genomic DNA. Concordance in the diagnoses of the 34 samples ranged from 91% (for *P. marinus*) to 97% (for both *H. costale* and *H. nelsoni*) (Table 6; Table S1 in the Supplement at www.int-res.com/articles/suppl/d151p111_supp.xlsx). All of the samples that showed a different result between platforms had C_T values close to the detection limit for each of the parasites tested. Inter-assay variation in the C_T values between platforms was shown to be within limits (≤5%) for the detection of *P. marinus* (2.54 ± 1.72%), *H. nelsoni* (3.28 ± 2.07%), and *H. costale* (2.02 ± 1.65%) (Table S1). Intra-assay variation between replicate wells for an individual sample was ≤2.0% on either platform for all targets (Table S2).

Table 5. Reproducibility of amplification efficiencies between qPCR detection platforms. Efficiencies ranged from 91 to 106% in the detection of *Perkinsus marinus*, *Haplosporidium nelsoni*, and *H.costale*

	P. marinus		*H. nelsoni*		*H. costale*	
	R^2	Efficiency (%)	R^2	Efficiency (%)	R^2	Efficiency (%)
Roche	1.000	93	0.995	106	0.995	105
BioRad	0.981	91	0.998	91	0.999	94

3.3. Concordance between histology and multiplex qPCR

The overall concordance between histology and qPCR for *P. marinus*, *H. nelsoni*, and *H. costale* was 54, 57, and 87%, respectively (Table 7; Table S3). Lack of concordance was, with a few exceptions noted below, due to increased sensitivity of the qPCR as compared to histology (false negatives by histology).

Samples tested for *H. costale* showed the lowest discordance (13%) between histology and qPCR (Table 7) of the 3 parasites tested, with most samples testing negative by both methods (82%). For all 3 parasites, C_T values of the samples that were positive by both methods ranged from 15 to 36 (Table S3), spanning the range from light to moderate (for *H. costale*) or heavy (for *H. nelsoni* and *P. marinus*) infections. Eleven of these samples showed dual infections for *H. nelsoni* and *H. costale* as detected by histology.

For *P. marinus*, light infections were frequently the cause of discordances between histology and qPCR (Table S3). Out of 56 samples, 26 (46%) that were negative by histology had C_T values ranging from 34

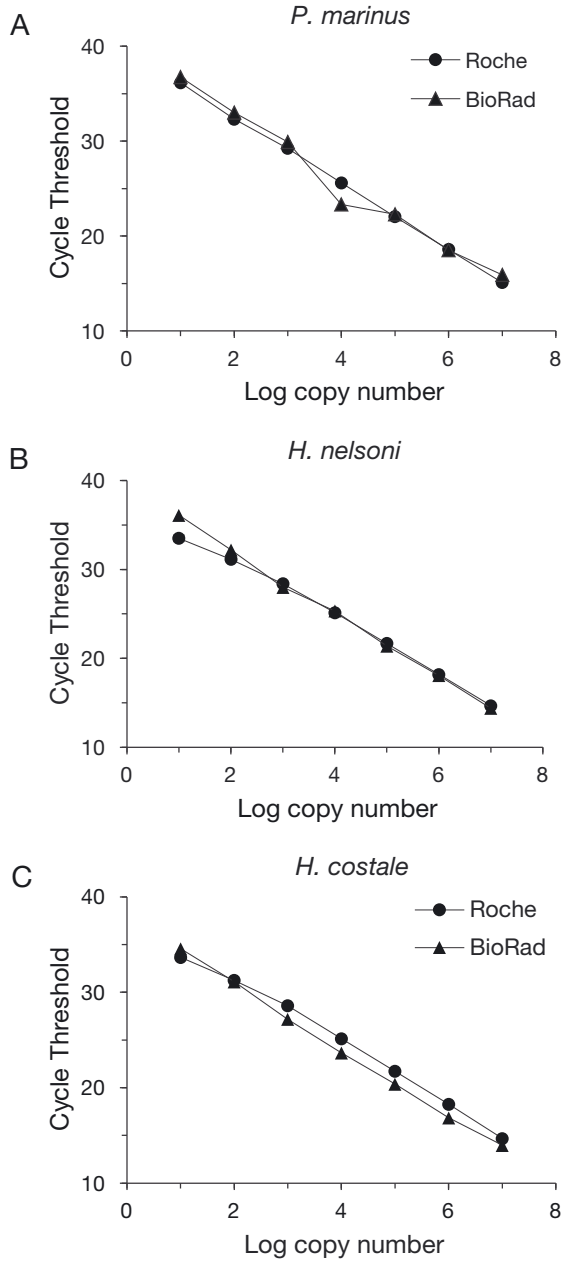

Fig. 2. Comparison of multiplex qPCR performance on 2 detection platforms. Standard curves from 2 multiplex runs performed on 2 different platforms (Roche Lightcycler® 480 vs. BioRad CFX96C1000). Assay sensitivity was higher on the BioRad platform with detection down to 10 copies of plasmid DNA for (A) *Perkinsus marinus*, (B) *Haplosporidium nelsoni*, and (C) *H. costale*, versus 100 copies of plasmid DNA for *H. nelsoni* and *H. costale* on the Roche platform

to 36 (less than 100 copies plasmid DNA), with the rest showing intermediate and advanced levels of *P. marinus* infection (C_t values ranging from 31 to 17, corresponding to 10^3 to 10^6 copies of *P. marinus* SSU). There were no samples that were positive by histology but negative by qPCR (Table 7).

Table 6. Concordance between Roche and BioRad platforms of samples tested by multiplex qPCR. Concordance is indicated by the number of samples in each category (positive by both platforms, negative by both platforms, etc.)/total number of samples tested (n = 34), followed by percent of samples in that category (in parentheses). The concordance between Roche and BioRad thermocyclers ranged from 91% (for *Perkinsus marinus*) to 97% (for both *Haplosporidium nelsoni* and *H. costale*)

Roche/BioRad	*P. marinus*	*H. nelsoni*	*H. costale*
Concordant +/+	18/34 (53)	19/34 (56)	6/34 (18)
Concordant –/–	13/34 (38)	14/34 (41)	27/34 (79)
Not concordant +/–	3/34 (9)	0/34 (0)	1/34 (3)
Not concordant –/+	0/34 (0)	1/34 (3)	0/34 (0)

Table 7. Concordance between multiplex qPCR and histology. Concordance is indicated by the number of samples in each category (positive by both methods, negative by both methods, etc.)/total number of samples tested (n = 121), followed by percent of samples in that category (in parentheses)

qPCR/Histology	*P. marinus*	*H. nelsoni*	*H. costale*
Concordant +/+	21/121 (17.4)	26/121 (21.5)	6/121 (5.0)
Concordant –/–	44/121 (36.4)	43/121 (35.5)	99/121 (81.8)
Not concordant +/–	56/121 (46.3)	47/121 (38.8)	2/121 (1.7)
Not concordant –/+	0/121 (0.0)	5/121 (4.1)	14/121 (11.6)

Light and intermediate infections (10^2–10^3 copies, C_T = 29–31) were also responsible for 43% (20 and 1, respectively) of the 49 discordant samples that were negative by histology for either *H. nelsoni* or *H. costale* but positive by qPCR for the corresponding parasite. Several samples negative by histology for either parasite (6 for *H. nelsoni* and 1 for *H. costale*, about 14% of the discordant samples) but positive by qPCR had a $C_T \leq 23$, showing that a small percentage of infections with 10^4–10^6 copies of either *H. nelsoni* or *H. costale* SSU were not detected by histology.

Interestingly, 14 samples were positive by histology and negative by qPCR for *H. costale* (Table 7). Of these 14 samples, 11 were positive for *H. nelsoni* by qPCR and also by histology, with heavy levels of infection, suggesting that, in this case, *H. nelsoni* was misidentified as *H. costale*. Conversely, the 3 samples that were positive by histology and negative by qPCR for *H. nelsoni* were also negative for *H. costale* by qPCR, so no *H. costale* samples were misidentified as *H. nelsoni* in this study.

Overall, based on qPCR detection, only 2 of the 121 samples (1.7%) were positive for all 3 parasites (*P. marinus, H. nelsoni,* and *H. costale*) (Table S3).

4. DISCUSSION

The multiplex qPCR assay was specific and sensitive at detecting infections with the protozoan parasites *Perkinsus marinus*, *Haplosporidium nelsoni*, and *H. costale* in the eastern oyster using 2 different detection platforms. The low variation in the efficiencies between targets on each platform demonstrated there was no significant competitive interference between the targets in the assay. Furthermore, we detected no PCR inhibition by the sample matrix (oyster tissue extracted with the Chelex method) using an internal amplification control. The performance of the multiplex in detecting *Haplosporidium* spp. was 10-fold more sensitive on the BioRad than the Roche platform, while *P. marinus* detection was equivalent for both platforms at 10 copies of target cloned plasmid DNA (SSU). The observed difference in the sensitivity between platforms suggests that some optimization of the assay should be performed when adapting the assay to different platforms. The dissociation curve analyses revealed some primer-dimer formation at low *H. nelsoni* and *H. costale* template concentrations. Primer-dimers reduce assay efficiency by competing for reaction mix components (Ruiz-Villalba et al. 2017). Further optimization to minimize these effects would increase the sensitivity of the assay in detecting low-level infections. Despite the reduced sensitivity of the assay with the Roche system, both platforms were in 91–97% agreement with the diagnoses of single and mixed infections, confirming the specificity of either platform in detecting *P. marinus*, *H. nelsoni*, or *H. costale*. Discordances between detection formats occurred only in samples that were at the lower end of the range of detection of the qPCR assay on either platform. Furthermore, the reproducibility of the assays was demonstrated by the low intra- and inter-assay variation (≤5%) between detection methods.

The sensitivity and specificity of the multiplex was evaluated using a diversity of methods. First, despite the increased diversity of haplosporidian species that has been recently documented through sequencing studies (Carnegie et al. 2014, Arzul & Carnegie 2015, Pagenkopp Lohan et al. 2016, Catanese et al. 2018, Davies et al. 2020, Lynch et al. 2020), the probes developed in this research showed identical matches for the whole length of the probe sequence with sequences for the targeted species, *H. costale* and *H. nelsoni*. Second, we compared the performance of the multiplex PCR against singleplex qPCR for *P. marinus* (De Faveri et al. 2009) and *H. nelsoni* (Wilbur et al. 2012), and traditional gel-based PCR for *H. costale* (Stokes & Burreson 2001) for a small number of samples. For *P. marinus* detection, 1 out of 5 samples deemed positive (C_T = 18) by singleplex was negative by multiplex qPCR on the BioRad platform. Since the 2 methods use the same primer/probe combination, potential reasons for this discordance include user/technical error or target degradation between the time the sample was first diagnosed using singleplex PCR and when it was tested again by multiplex qPCR. In regard to *H. nelsoni* infection in oysters, the singleplex and multiplex assays showed a similar sensitivity and specificity, with disagreements between detection formats only occurring in a sample that was at the lower end of the range of detection of the qPCR assay (i.e. light infections). These data suggest that multiplexing decreases the sensitivity of this assay in detecting *H. nelsoni*. In the case of detecting *H. costale* infection in oysters, the multiplex qPCR assay was as sensitive as traditional gel-based PCR for SSO detection on both qPCR platforms. The identical performance between detection formats suggests that the sensitivity and specificity were retained even when multiplexed.

We also compared the performance of the multiplex qPCR to histology. A relatively low level of concordance was seen between histology and the multiplex qPCR. The majority of the samples showing a lack of concordance between methods were positive by multiplex qPCR for *P. marinus* and *H. nelsoni* but negative by histology, suggesting that the multiplex assay was more sensitive at detecting *P. marinus* and *H. nelsoni* infections. It has been well documented that PCR or qPCR methods are more sensitive than histology, with histological inaccuracies occurring in samples with low to moderate levels of (most likely localized) infections (Marty et al. 2006, Wilbur et al. 2012). The relatively high frequency of false negatives as detected by histology is proposed to be due to the inherent difficulties of distinguishing single cells from small protozoan parasites within oyster tissues. Early single nucleated/spore forms of *P. marinus, H. nelsoni*, and *H. costale*, especially when dispersed through tissues and not aggregated within an area, are notoriously difficult to positively identify in histological sections. Several of the samples that were negative by histology but positive by qPCR, however, showed C_T values consistent with moderate or heavy infections. In this case, lack of detection by histology

could also be a consequence of tissue sampling procedures, as only a small thin section including the mantle and gill is evaluated by histology, or due to the occurrence of single infectious cells in various locations of the tissues (not identifiable by histology). Limitations from the tissue sampling procedures in detecting low levels of very localized infections can also lead to false negatives in the case of qPCR/PCR testing, since small pieces of tissue are collected, processed, and tested in these analyses, mainly to avoid PCR inhibition by the matrix (Wilbur et al. 2012).

It is probable that the lack of concordance between histology and qPCR in the case of *H. costale* was a result of the misidentification of the *Haplosporidium* parasites in histology samples, due to the difficulty in distinguishing morphological variations between species (Andrews & Castagna 1978, Stokes & Burreson 2001). For example, in this study, there were 14 samples that were positive by histology for *H. costale* but negative by qPCR. Of these, 11 were also positive for MSX by qPCR and identified as heavily infected by histology, suggesting that the multinucleated and/or the single-cell form belonging to *H. nelsoni* were misidentified as *H. costale*. These results confirm that qPCR assays are more sensitive at detecting and distinguishing between *Haplosporidium* spp. than traditional visual methods for diagnosis by histology. The greater sensitivity and specificity of the qPCR minimizes the need for expertise in distinguishing histomorphological differences (Stokes & Burreson 2001, Xie et al. 2013). Moreover, this multiplex qPCR provides an advantage over a previously described duplex qPCR assay that does not allow for the differentiation between these 2 *Haplosporidium* spp. without further analyses (Xie et al. 2013).

In conclusion, we have developed a multiplex assay for the simultaneous detection of 3 protozoan parasites that have significant economic and ecological impacts on eastern oyster health in a single reaction. This assay has the advantage of being more sensitive and less time consuming than other diagnostic methods, further allowing for the specific detection of 2 related *Haplosporidium* spp. that are not easily differentiated using histological techniques. Implementing molecular diagnostics with species-specific primers and probes in routine oyster disease monitoring will increase the specificity and sensitivity of detection as well as allow for a more rapid and corrective response.

Acknowledgements. This research was supported by funding from the USDA ARS (CRIS project no. 80303100000300D). Live oysters or oyster genomic DNA for use in the multiplex qPCR validation were kindly donated by Ami Wilbur (University of North Carolina at Wilmington), Susan Ford (Haskin Shellfish Research Laboratory), Inke Sunila (State of Connecticut Department of Agriculture), Paul Rawson and Timothy Bowden (University of Maine), and Cem Giray (Kennebec River Biosciences). Special thanks to Inke Sunila for providing valuable feedback on the paper.

LITERATURE CITED

Andrews JD, Castagna M (1978) Epizootiology of *Minchinia costalis* in susceptible wild oysters in Virgnia's eastern shore, 1959–1976. J Invertebr Pathol 32:124–138

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

Aranishi F, Okimoto T (2006) A simple and reliable method for DNA extraction from bivalve mantle. J Appl Genet 47:251–254

Arzul I, Carnegie RB (2015) New perspective on the haplosporidian parasites of molluscs. J Invertebr Pathol 131: 32–42

Arzul I, Garcia C, Chollet B, Serpin D and others (2022) First characterization of the parasite *Haplosporidium costale* in France and development of a real-time PCR assay for its rapid detection in the Pacific oyster, *Crassostrea gigas.* Transbound Emerg Dis 69:e2041-e2058

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

Barber BJ, Ford SE, Haskin HH (1988) Effects of the parasite MSX (*Haplosporidium nelsoni*) on oyster (*Crassostrea virginica*) energy metabolism. I. Condition index and relative fecundity. J Shellfish Res 7:25–31

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

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

Carnegie RB, Hill KM, Stokes NA, Burreson EM (2014) The haplosporidian *Bonamia exitiosa* is present in Australia, but the identity of the parasite described as *Bonamia* (formerly *Mikrocytos*) *roughleyi* is uncertain. J Invertebr Pathol 115:33–40

Carnegie RB, Arzul I, Bushek D (2016) Managing marine mollusc diseases in the context of regional and international commerce: policy issues and emerging concerns. Philos Trans R Soc B 371:20150215

Carnegie RB, Ford SE, Crockett RK, Kingsley-Smith PR and others (2021) A rapid phenotype change in the pathogen *Perkinsus marinus* was associated with a historically significant marine disease emergence in the eastern oyster. Sci Rep 11:12872

Catanese G, Grau A, Valencia JM, Garcia-March JR and others (2018) *Haplosporidium pinnae* sp. nov., a haplosporidan parasite associated with mass mortalities of the fan mussel, *Pinna nobilis*, in the Western Mediterranean Sea. J Invertebr Pathol 157:9–24

CRMC (Coastal Resources Management Council) (2012) Minutes of the CRMC BioSecurity Board. www.crmc.ri.gov/meetings/2012_0119biosec2.html

Davies CE, Bass D, Ward G, Batista F and others (2020) Diagnosis and prevalence of two new species of haplosporidians infecting shore crabs *Carcinus maenas*: *Haplosporidium carcini* n. sp., and *H. cranc* n. sp. Parasitology 147:1229–1237

De Faveri J, Smolowitz RM, Roberts SB (2009) Development and validation of a real-time quantitative PCR assay for the detection and quantification of *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica*. J Shellfish Res 28:459–464

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797

Espy MJ, Uhl JR, Sloan LA, Buckwalter SP and others (2006) Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev 19:165–256

Farley CA (1968) *Minichinia nelsoni* (Haplosporidia) disease syndrome in the American oyster *Crassostrea virginica*. J Protozool 15:585–599

Ford SE (2002) Development of high disease resistance in a wild oyster population. J Shellfish Res 21:387

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, Tripp MR (1996) Diseases and defense mechanisms. In: Kennedy VS, Newell RIE, Eble AF (eds) The eastern oyster *Crassostrea virginica*. Maryland Sea Grant College, College Park, MD, p 581–660

Ford SE, Paterno J, Scarpa E, Stokes NA, Kim Y, Powell EN, Dushek D (2011) Widespread survey finds no evidence of *Haplosporidium nelsoni* (MSX) in Gulf of Mexico oysters. Dis Aquat Org 93:251–256

Haskin HH, Stauber LA, Mackin JA (1966) *Minchinia nelsoni* n. sp. (Haplosporidia, Haplosporidiidae): causative agent of the Delaware Bay oyster epizootic. Science 153: 1414–1416

Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P (2004) Practical considerations in design of internal amplification controls for diagnostic PCR assays. J Clin Microbiol 42:1863–1868

Howard DW, Lewis EJ, Keller BJ, Smith CS (2004) Histological techniques for marine bivalve mollusks and crustaceans. Tech Memo NOS NCCOS 5. NOAA, Oxford, MD

ICES (2020) Working group on pathology and diseases of marine organisms (WGPDMO). International Council for the Exploration of the Sea (ICES) Sci Rep 2. ICES, Copenhagen

Ko YT, Ford SE, Fong D (1995) Characterization of the small subunit ribosomal RNA gene of the oyster parasite *Haplosporidium costale*. Mol Mar Biol Biotechnol 4:236–240

López-Sanmartín M, Catanese G, Grau A, Valencia JM, García-March JR, Navas JI (2019) Real-Time PCR based test for the early diagnosis of *Haplosporidium pinnae* affecting fan mussel *Pinna nobilis.* PLOS ONE 14: e0212028

Lynch SA, Lepée-Rivero S, Kelly R, Quinn E and others (2020) Detection of haplosporidian protistan parasites supports an increase to their known diversity, geographic range and bivalve host specificity. Parasitology 147:584–592

Marty GD, Bower SM, Clarke KR, Meyer G and others (2006) Histopathology and a real-time PCR assay for detection of *Bonamia ostreae* in *Ostrea edulis* cultured in western Canada. Aquaculture 261:33–42

Menzel RW, Hopkins SH (1955) Growth of oysters parasitized by the fungus *Dermocystidium marinum* and by the trematode *Bucephalus cuculus.* J Parasitol 41: 333–342

Myhre JL, Haskin HH (1967) Some observations on the development of early *Minchinia nelsoni* infections in *Crassostrea virginica* and some aspects of the host–parasite relationship. Proc Natl Shellfish Assoc 58:7

Nordstrom JL, Vickery MCL, Blackstone GM, Murray SL, DePaola A (2007) Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. Appl Environ Microbiol 73: 5840–5847

OIE (2021) Aquatic animal health code 2021, 23rd edn. World Organisation for Animal Health, Paris

Pagenkopp Lohan KM, Hill-Spanik KM, Torchin ME, Aguirre-Macedo L, Fleischer RC, Ruiz GM (2016) Richness and distribution of tropical oyster parasites in two oceans. Parasitology 143:1119–1132

Perkins FP (1969) Electron microscope studies of sporulation in the oyster pathogen, *Minchinia costalis* (Sporozoa; Haplosporidia). J Parasitol 55:897–920

Ragone Calvo LM, Calvo GW, Burreson EM (2003) Dual disease resistance in a selectively bred eastern oyster, *Crassostrea virginica*, and strain tested in Chesapeake Bay. Aquaculture 220:69–87

Ray SM (1952) A culture technique for the diagnosis of infections with *Dermocystidium marinum* Mackin, Owen, and Collier in oysters. Science 116:360–361

Ray SM, Mackin JG, Boswell JL (1953) Quantitative measurement of the effect on oysters of disease caused by *Dermocystidium marinum.* Bull Mar Sci Gulf Caribb 3: 6–33

Robledo JAF, Gauthier JD, Coss CA, Wright AC, Vasta GR (1998) Species-specificity and sensitivity of a PCR-based assay for *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica*: a comparison with the fluid thioglycollate assay. J Parasitol 84:1237–1244

Roche Life Technologies (2012) Real time PCR handbook. https://www.gene-quantification.de/real-time-pcr-handbook-life-technologies-update-flr.pdf

Rodríguez A, Rodríguez M, Andrade MJ, Cordoba JJ (2012) Development of a multiplex real-time PCR to quantify aflatoxin, ochratoxin A and patulin producing molds in foods. Int J Food Microbiol 155:10–18

Ruiz-Villalba A, van Pelt-Verkuil E, Gunst QD, Ruijter JM, van den Hoff MJB (2017) Amplification of nonspecific products in quantitative polymerase chain reactions (qPCR). Biomol Detect Quantif 14:7–18

Soudant P, Chu EFL, Volety A (2013) Host–parasite interactions: marine bivalve molluscs and protozoan parasites, *Perkinsus* species. J Invertebr Pathol 114:196–216

Stokes NA, Burreson EM (2001) Differential diagnosis of mixed *Haplosporidium costale* and *Haplosporidium nelsoni* infections in the eastern oyster, *Crassostrea virginica*, using DNA probes. J Shellfish Res 20: 207–213

Stokes NA, Siddall ME, Burreson EM (1995) Detection of *Haplosporidium nelsoni* (Haplosporidia: Haplosporidiidae) in oysters by PCR amplification. Dis Aquat Org 23: 145–152

Sunila I, Karolus J, Volk J (1999) A new epizootic of Haplosporidia (MSX), a haplosporidian oyster parasite, in Long Island Sound. J Shellfish Res 18:169–174

Villalba A, Reece KS, Ordas MC, Casas SM, Figueras A (2004) Perkinsosis in molluscs: a review. Aquat Living Resour 17:411–432

Wilbur AE, Ford SE, Gauthier JD, Gomez-Chiarri M (2012) Quantitative PCR assay to determine prevalence and intensity of MSX (*Haplosporidium nelsoni*) in North Carolina and Rhode Island oysters *Crassostrea virginica*. Dis Aquat Org 102:107–118

Wood JL, Andrews JD (1962) *Haplosporidium costale* (Sporozoa) associated with a disease of Virginia oysters. Science 136:710–711

Xie Z, Xie L, Fan Q, Pang Y and others (2013) A duplex quantitative real-time PCR assay for the detection of *Haplosporidium* and *Perkinsus* species in shellfish. Parasitol Res 112:1597–1606

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

Editorial responsibility: Kimberly Reece, Gloucester Point, Virginia, USA
Reviewed by: S. Gignoux-Wolfsohn, S. Fernández-Boo and 1 anonymous referee

Submitted: May 10, 2022
Accepted: August 15, 2022
Proofs received from author(s): October 14, 2022