Critical assessment of baseline disease dynamics of natural oyster beds in Long Island Sound – establishing foundational knowledge to inform oyster restoration and aquaculture disease management.

Participating organizations: NOAA, Northeast Fisheries Science Center, Milford Laboratory

Coordinating organization: NOAA, Northeast Fisheries Science Center, Milford Laboratory

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Date approved:

Completed Plan Prepared By:
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1. PROJECT MANAGEMENT

1.1 Contact Information

Laboratory work will be performed at the NOAA Milford Lab, 212 Rogers Ave, Milford, CT, 06460. Contact information for key project team personnel and the general organizational structure for key leads on the project are listed in **Table 1–1** and the following text. All volunteers and interns will work at the direction of key project team personnel.

All personnel listed below in **Table 1–1** will receive copies of this Quality Assurance Project Plan (QAPP), and any approved revisions of this plan. Once approved, this QAPP will be available to any interested party by requesting a copy from the project management.

Table 1–1. Project team contact information

Title	Name, Affiliation	E-mail
Project Manager/QA Manager (Field data)	Meghana Parikh, NEFSC Milford	meghana.parikh@noaa.gov
Project Manager/QA Manager (Lab data)	Katherine McFarland, NEFSC Milford	katherine.m.mcfarland@noaa.gov
Field Lead	Steve Arnott, NEFSC Milford	stephen.arnott@noaa.gov
Field/Lab Technician	Mariah Kachmar, NEFSC Milford	mariah.kachmar@noaa.gov
Milford Lab Director	Gary Wikfors, NEFSC Milford	gary.wikfors@noaa.gov
Sea Grant Extension Specialist	Zach Gordon, Connecticut Sea Grant & NEFSC Milford	zachary.gordon@noaa.gov
Dive Coordinator	Barry Smith, NEFSC Milford	barry.smith@noaa.gov
Connecticut State Shellfish Pathologist	Lydia Bienlien, Connecticut Department of Agriculture, Bureau of Aquaculture	lydia.bienlien@ct.gov
EPA Project Officer	Casey Abel, EPA Region 1	Abel.Casey@epa.gov
QAPP Reviewer	Elise McNally, EPA Region 1	McNally.Elise@epa.gov

PROJECT MANAGERS (Meghana Parikh and Katherine McFarland) have the overall responsibility for ensuring that the project meets the project objectives and quality standards. The Project Managers will be responsible for overseeing all activities conducted on this project including schedule adherence, budgeting, and oversight of all scope-related activities. Scope-related activities include assigning project tasks to personnel, data collection, data analysis, interpretation, communication, and final reporting. The Project Managers will also

coordinate all program/project needs related to project personnel and convene periodic project-planning meetings. The laboratory portion of tissue analysis will be led and overseen by Meghana Parikh and the field portion of data collection will be led and overseen by Katherine McFarland.

QA MANAGERS (Meghana Parikh and Katherine McFarland) will be responsible for overseeing all data entry, data QA/QC, and final archiving of data collected throughout the duration of the project. The responsibilities of QA manager will be split between to lead PI's. QA/QC of laboratory data will be led and overseen by Katherine McFarland and the QA/QC of field data will be led and overseen by Meghana Parikh. Further details of QA Manager activities are included in **Sections 4.2, 6.1,** and **7**. Meghana Parikh will assume the overall responsibility for ensuring that the data meets the project objectives and quality standards outlined in the QAPP through assessments (**Section 7**) and will ensure that corrective actions are implemented, if necessary.

FIELD LEAD (Stephen Arnott) has the overall responsibility of site coordination and logistical planning for monthly site visits. Scope-related activities include coordination with site hosts, local authorities (e.g., shellfish commissions), and all staff and volunteers. The field lead will oversee the execution of field activities, ensuring adherence to field data collection protocols. The field lead will also prepare materials for the field, organize and delegate field tasks to personnel, and ensure that personnel have the necessary resources and training to conduct the field activities safely.

FIELD/LAB TECHNICIAN (Mariah Kachmar) has the overall responsibility of assisting the field lead with all field related activities and will lead laboratory sample processing and analysis. Scope-related activities include sample processing, storage, analysis, and data entry of laboratory collected data. The field/lab technician will also maintain inventory of required laboratory supplies and will assist with compliance with laboratory health and safety protocols.

LAB DIRECTOR (Gary Wikfors) has the overall responsibility of ensuring adequate lab spaces are available and safe for operation. Scope-related activities include ensuring compliance with all US Department of Commerce, NOAA Fisheries safety, security, and information technology requirements.

SEA GRANT EXTENSION SPECIALIST (Zach Gordon) has the overall responsibility of communicating with local shellfish and community outreach to keep local stakeholders informed of the research. As a NOAA Certified Diver, the Sea Grant Extension Specialist may also assist with field data collection at subtidal locations.

DIVE COORDINATOR (Barry Smith) has the overall responsibility of coordinating all dive logistics for subtidal field sites. Scope-related activities include organizing a qualified dive team and assuring all associated gear is held to all safety standards. The dive coordinator will also be responsible for certifying all new team members to be NOAA approved and coordinate required training.

CONNECTICUT STATE SHELLFISH PATHOLOGIST (Lydia Bienlien) has the overall responsibility of assisting with histological analysis of disease pathology and reproductive status. Scope-related activities include coordinating the use of the State pathology lab to process histology samples and prepare slides, guidance on best practices, and assistance with scoring infection and reproduction indices.

LABORATORY INFORMATION

Name NOAA MIlford Laboratory	
Address 212 Rogers Ave., Milford, CT 06460	
Phone (203) 882-6500	**Contact Name** Gary H. Wikfors (Lab Director)
Organization/Laboratory Certification No. Registered Laboratory Utilizing Living Agents for Teaching, Research, and/or Quality Control (BSL-2) - State of Connecticut Department of Public Health. License #1204	**Expiration Date** 03/31/2024

Name State of Connecticut, Department of Agriculture, Bureau of Aquaculture	
Address 190 Rogers Ave., Milford, CT 06460	
Phone (203) 874-0696 x120	**Contact Name** Lydia Bienlien (State Bivalve Shellfish Pathologist)
Organization/Laboratory Certification No. N/A	**Expiration Date**

In addition to the core project team and facilities outlined above, numerous state, local, and non-profit organizations are providing input to select suitable study sites and garner support to execute this work from nearby communities. These groups are described in more detail in **Section 1.2.4** and **Table 1–4**.

1.2 Project Objectives and Approach

1.2.1 Background / Project Rationale

The EPA Long Island Sound Study (LISS) has been remarkably effective in identifying and supporting achievement of environmental goals related to human uses of the Long Island Sound (LIS), a body of water with enormous economic and cultural value often referred to as a national treasure. Perhaps the major accomplishment of the LISS has been to recognize and address the unique challenges of nutrient management in LIS. More than a decade ago, LISS and NOAA's Northeast Fisheries Science Center (NEFSC) and National Center for Coastal Ocean

Science (NCCOS) initiated modest, ongoing work supporting the idea of harnessing the nutrient-accumulating activities of bivalve shellfish and macroalgae for this purpose. Increased national investment in LISS has presented the opportunity to accelerate this research to more-fully realize the potential contributions of bivalve shellfish, farmed, restored, and naturally-occurring, to water quality and habitat provisioning in LIS. Our work seeks to preserve and maximize the contributions of the existing shellfish (primarily oyster) industry and to build upon this foundation by informing the active management of natural, restored, and cultivated populations for a full array of ecosystem services.

A critical first step to successfully restoring and managing bivalve populations is to understand the current environment and status of the desired restoration sites (Fitzsimons et al., 2019). Currently, the Connecticut portion of Long Island Sound has had little shellfish restoration with exception of the public natural beds used as a source for oyster seed for the aquaculture industry, but maintains several distinct populations of naturally occurring oyster beds throughout its tributaries and coastal waters (Sunila et al., 2016). Expanding existing beds may present a desirable opportunity to expand population densities and oyster habitat coverage, but little is known about disease prevalence and intensity within these populations. Recent restoration efforts on the New York side of Long Island Sound present similar opportunities and challenges. The spread and proliferation of MSX disease led to a near extirpation of Connecticut oysters, both farmed and natural, in 1997 (Sunila et al., 1999; Sunila et al., 2016). Hatchery based production of seed (juvenile oysters) supplemented the aquaculture industry and in recent years, naturally occurring, self-sustaining oyster beds have been identified and utilized as a natural seed source by the oyster farming industry. At the same time, nearby state-designated shellfish beds have not seen similar seed production, leading to questions about water quality and habitat criteria for healthy oyster reproduction and survival. While significant work by local agencies to expand these beds has increased natural seed production and provided extensive ecosystem services, concern regarding the amplification of shellfish parasites causing Dermo (*Perkinsus marinus*), MSX (*Haplosporidium nelsoni*), and SSO (*Haplosporidium costale* diseases is an important consideration to the aquaculture industry. Shellfish growers have largely been able to reduce pathogen prevalence and avoid mortalities by harvesting before the fatal progression of disease after two years of infection. There is concern that in the absence of active management, increased oyster population density from restoration efforts will intensify the disease burden on all oysters in the Sound, and subsequently decrease the biomass and ecosystem services provided by natural, restored, and aquacultured shellfish in LIS (CT DABA, pers. comm.).

To develop risk-based guidance for mitigating disease in restored populations, greater understanding is needed about the presence and progression of disease in thriving, unmanaged populations. These natural beds serve as a unique model of the potential structure of stable, successfully-restored oyster populations, and this offers valuable perspective on the burden of disease we may see in future restoration projects. We propose to establish baseline measurements of disease prevalence and reproductive fitness of natural, established oyster populations, as well as presently and previously active restoration sites in LIS. Measures of water quality, oyster-bed structure, population demographics, reproductive fitness, and juvenile

recruitment will be recorded alongside health and disease data to identify key environmental and biological characteristics associated with disease burdens in the studied populations. Tissue samples will be collected and preserved for future projects characterizing population genetic structure to identify genotypes associated with disease tolerance. Additionally, we will collect continuous water quality data (temperature, salinity, dissolved oxygen, pH, and chlorophyll a) to better characterize habitat-specific factors affecting oyster population health and disease progression. These data are often sparse in LIS and continuous water quality monitoring would benefit many LISS Comprehensive Conservation and Management Plan goals and benefit research to improve LIS water quality through habitat restoration and shellfish populations more broadly. By partnering with LIS stakeholders we will synthesize the new data with existing disease surveillance data on aquacultured oyster populations and water quality measures captured through other existing LISS projects. This work will provide foundational knowledge about disease intensity, prevalence, and progression in natural and restored unmanaged (unmanaged = no harvest) oyster beds that is currently lacking in the scientific literature. Pairing these data with population structure, reproductive fitness, juvenile recruitment, and water quality data will provide information on the abiotic and biotic factors that affect disease spread and proliferation. These data will provide baseline disease and health metrics essential to restoration and aquaculture activities in LIS.

1.2.2 Objectives

OBJECTIVE 1: Characterize population health and stability of natural and restored Eastern oyster populations in Long Island Sound on seasonal and annual timescales by measuring infection prevalence and intensity, body condition, and reproductive success of individual oysters within four unharvested populations in LIS.

OBJECTIVE 2: Identify the key water quality parameters and oyster population dynamics associated with observed burdens of disease and reproductive fitness in the study populations using continuous water monitoring tools, oyster bed characterization, and oyster population structure.

1.2.3 Approach and timeline

The project will sample, monitor, and analyze data from four oyster beds in Long Island Sound. All four oyster beds will be in an unharvested state to ensure that a full range of oyster life history stages are present, including large adults, which is the stage most likely to be affected by diseases (Andrews, 1988; Andrews, 1982). The project will include the following field and laboratory components:

FIELD WORK

- Oyster bed water quality (water temperature, depth, salinity, pH, dissolved oxygen, chlorophyll *a*)
- Oyster bed characteristics (area of coverage, elevation/depth, bed height, rugosity)
- Population demographics (percent cover, density, mortality, size composition)
- Recruitment of newly settled juvenile oysters, hereafter, spat (with lab-verification of settled spat)

- Growth and survival of juvenile oysters
- Sampling of adult oysters for laboratory analyses

LABORATORY WORK

- Preparing adult oyster specimens for tissue sampling
- Sampling adult oyster tissues for disease and reproductive assays
- Disease Diagnostics: Triplex qPCR assay for pathogen detection
- Disease Diagnostics: Ray's Fluid Thioglycollate Medium for detection of *P. marinus*
- Disease Diagnostics: Histology for pathogen detection and diagnosis of disease
- Reproductive assessment of adult oysters
- Archiving tissue for genomic analysis

The work will cover a two year period, with field sampling occurring from March–November of 2023 and 2024. The timescale of project components is shown in **Table 1–2**.

Table 1–2. Monthly timing (shaded cells) of field and laboratory components of the project during 2023 and 2024.

		2023												2024											
	Project component	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D
FIELD	Water temperature																								
	Water depth, salinity, pH, dissolved oxygen, chlorophyll *a*																								
	Oyster bed characteristics (area, elevation/depth, bed height, rugosity)																								
	Population demographics (% cover, survival, density, size composition)																								
	Recruitment of oyster spat																								
	Growth and survival of juvenile oysters																								
	Sampling of adult oysters for lab analyses																								
LAB	Preparing oysters for tissue sampling																								
	Sampling oyster tissues for pathogen detection and reproductive assays																								
	Disease Diagnostics: qPCR																								
	Disease Diagnostics: RFTM																								
	Disease Diagnostics: Histology																								

Table 1–3. Responsibility assignment matrix showing *primary* roles of project staff. R: responsible; A: accountable; C: consulted; I: informed.

Task	Type	Parikh	McFarland	Arnott	Kachmar
Water quality	Field	C	A	R	R
Oyster bed characteristics	Field	C	A	R	R
Population demographics	Field	C	A	R	R
Oyster recruitment and growth	Field	C	A	R	R
Preparing adult oysters for tissue sampling	Lab	A	C	R	R
Tissue sampling of adult oysters	Lab	A	C	R	R
Molecular disease diagnostics	Lab	A	C	I	R
Microscopical disease diagnostics	Lab	A	C	I	R
Histological disease diagnostics	Lab	A	C	I	R
Histological reproductive assessment	Lab	C	A	I	R
QA/QC of field data	Field	C	A	I	R
QA/QC of lab data	Lab	A	C	R	I

1.2.4 Study Sites

Four oyster beds will be selected, two in Connecticut and two in New York. To be included in the study, the site needs to meet the following criteria:

- Presence of a dense oyster population, defined as being substantial enough that individual oysters are touching or growing on-top of others.
- Oysters growing on natural substrate (e.g., natural sand, shell, rock, or oyster restoration substrate) independent of structures intended for human use (e.g., dock, pilings, support pillars).
- The oyster bed can be naturally occurring, or the result of restoration efforts.
- Active restoration, where either shell or whole oysters are planted, may occur during the study timeframe. If these activities occur, they will be carefully documented to improve interpretation of oyster biometric data and oyster bed structure data.
- Closed to commercial and recreational harvest in the past 5 years and throughout the study timeframe. Accurate characterization of population disease dynamics is dependent on the inclusion of oysters matured beyond the usual aquaculture harvest timeline (~2-3 years).
- There should be no plans to dredge the bed during the study timeframe.

Table 1–4 details four tentative study areas. These areas were identified with input from state, local, and non-profit shellfish and conservation programs. These groups include the Connecticut Department of Agriculture, Bureau of Aquaculture (DABA), Connecticut Sea Grant (CTSG), New York State Department of Environment and Conservation (NYSDEC), Cornell Cooperative Extension (CCE), New York Sea Grant, Fairfield Shellfish Commission, Town of Fairfield, Ash Creek Conservation Association, Clinton Shellfish Commission, Indian River Shellfish Company, Oyster Bay Cold Spring Harbor Protection Committee, and Adelphi University. All relevant partners have been contacted and we have support to proceed with our work at the sites listed here. Precise GPS coordinates of the oyster beds are still being determined with the help of the aforementioned local officials and partners. Final decisions about study site locations and monitoring methods will be made according to the guidelines set forth in this QAPP after we are able to visually inspect the tentative sites.

Table 1–4. Tentative study sites for monitoring unmanaged oyster populations.

Site Name, Town	**Ash Creek, Fairlfield**	**Fence Creek, Madison**	**Gold Star Battalion Beach, Huntington**	**Cold Spring Harbor, Oyster Bay**
State	Connecticut	Connecticut	New York	New York
Partners/Local Officials	Fairfield Shellfish Commission, Town of Fairfield, Ash Creek Conservation Association, DABA, CTSG	Madison Shellfish Commission, Indian River Shellfish Company, DABA, CTSG, NYSG	CCE, Town of Huntington, NYSDEC, NYSG	CCE, Town of Oyster Bay, Friends of the Bay, Cold Spring Harbor Protection Committee, Adelphi University, NYSDEC
Intertidal or Subtidal	Intertidal	Intertidal	Subtidal	Subtidal
Natural or Restored	Restored	Natural	Restored	Restored
Active or Historical Restoration	Active – adding shell base and juvenile oysters	NA	Active – planting juvenile oysters	Active – planting juvenile oysters
Recreational Harvest	Prohibited	Prohibited	Prohibited	Prohibited

* Determination of exact coordinates of the oyster bed is still underway in this region.

Figure 1–1. Approximate locations of tentative study sites within New York and Connecticut portions of Long Island Sound.

1.3. Data Quality Objectives

1.3.1 Water Quality

Water quality parameters will be collected using continuous monitoring, deployable Aqua Troll 600 data sondes at each site. Main water quality parameters that will be targeted are Temperature, Salinity, Dissolved Oxygen, pH, and Chlorophyll a. This characterization of the local environment will allow for a better understanding of how environmental conditions affect disease progression and aid in model projections.

Table 1–5. Water quality and associated parameters to be measured.

Parameter	**Unit**
Water temperature	degrees Celsius (°C)
Salinity	no unit

Dissolved Oxygen	milligrams/liter (mg/L)
pH	no unit
Chlorophyll-a	Relative fluorescence units (RFU)

1.3.2 Oyster Bed Characteristics

Table 1–6. Data quality objectives for quantifying oyster bed characteristics

Parameter	**Unit**
Area of oyster bed	Square meters
Tidal elevation (intertidal sites only)	At, or lower than, mean low water
Minimum and maximum depth below mean low water (subtidal sites only)	Meters
Rugosity index	Contoured length divided by straight-line distance (no unit)

1.3.3 Population demographics

Table 1–7. Data quality objectives for Population demographics.

Population Parameter	**Unit**
Density of live oysters	Number of live oysters / meter squared
Density of dying and moribund oysters	Number of gaper oysters / meter squared
Density of recently dead adult oysters (empty articulated shells with no tissue)	Number of box oysters / meter squared
Gaper mortality	$\frac{Number\ of\ gaper\ oysters\ per\ meter\ squared}{Number\ of\ live + box + gaper\ oysters\ per\ meter\ squared}$%
Total mortality	$\frac{Number\ of\ box + gaper\ oysters\ per\ meter\ squared}{Number\ of\ live + box + gaper\ oysters\ per\ meter\ squared}$%
Shell height	mm
Shell length	mm
Size composition of oysters	Percent of population per 10 mm shell height category.

1.3.4 Oyster recruitment, juvenile survival, and growth

Table 1–8. Oyster spat recruitment, growth, and survival parameters to be measured.

Parameter	Unit
Spat recruitment	Number of oyster spat per shell
Survival	Percent survival per month
Shell growth (change in shell height per month)	mm per month

1.3.5 Preparing oysters for tissue sampling

Table 1–9. Data collected during oyster tissue sampling.

Parameter	Unit
Total weight	grams
Shell height	mm
Shell length	mm
Shell width	mm
Tissue condition	Fat, medium, watery (categorical)
Gross internal lesions / general observations	Free text (no unit)

1.3.6 Sampling oyster tissues for pathogen detection and reproductive assays

No data will be recorded during tissue dissection, except for specimen ID (for labeling and specimen tracking) and confirmation of successful sampling (check-off list of specimen ID and tissue type).

1.3.7 Disease Diagnostics: Triplex qPCR assay for pathogen detection

Table 1–10. Data collected for each individual specimen to characterize disease intensity and prevalence using the newly developed triplex qPCR method for all three parasites (*P. marinus*, *H. nelsoni*, *H. costale*).

Parameter	Target(s)	Unit
Infection intensity (individual)	*P. marinus*	qPCR-Mackin Scale (Roger Williams University, pers. comm.)

		DNA copy number → Mackin Score <10 = 0 10 - 100 = 0.5 100 - 2,000 = 1 2,000 - 10,000 = 2 10,000 - 100,000 = 3 100,000 - 1,000,000 = 4 >1,000,000 = 5
Infection intensity (individual)	*H. nelsoni* *H. costale*	qPCR-Mackin Scale (Roger Williams University, pers. comm.) DNA copy number → Intensity Score <10 = 0 10-10,000 = 1 10,000 - 1,000,000 = 2 <1,000,000 = 3
Prevalence (population)	*P. marinus* *H. nelsoni* *H. costale*	% (# of positive individuals / # of sampled individuals)
Weighted Prevalence (population)	*P. marinus* *H. nelsoni* *H. costale*	(Sum of intensity scores / # of all sampled individuals)

1.3.8 Disease Diagnostics: Ray's Fluid Thioglycollate Medium for detection of *P. marinus*

Table 1–11. Data collected for each individual specimen to characterize disease intensity and prevalence using classic methods for *P. marinus*.

Parameter	**Unit**
P. marinus Infection intensity (individual)	Mackin Scale: 0-5 (semi-quantitative) (Ray,1954a, 1954b, Mackin, 1962)
P. marinus Prevalence (population)	% (# of positive individuals / # of sampled individuals)
P. marinus Weighted Prevalence (population)	(Sum of intensity scores / # of all sampled individuals)

1.3.9 Disease Diagnostics: Histology for pathogen detection and diagnosis of disease

Table 1–12. Data collected for each individual specimen to characterize disease intensity and prevalence using classic histological methods for all three parasites, *P. marinus*, *H. nelsoni* and *H. costale*.

Parameter	Target(s)	Unit
Infection intensity (individual)	*P. marinus*	Scoring 0–5 (semi-quantitative) (Mann et al., 2014)
Infection intensity (individual)	*(H. nelsoni & H. costale)**	Scoring 0–4 (categorical) (Carnegie & Burreson, 2011)
Prevalence (population)	*P. marinus*; *(H. nelsoni & H. costale)**	% (# of positive individuals / # of sampled individuals)
Weighted Prevalence (population)	*P. marinus*; *(H. nelsoni & H. costale)**	(Sum of intensity scores / # of all sampled individuals)

**H. nelsoni and H. costale* are indistinguishable via histology, so both *Haplosporidium* spp. are included in reported metrics.

1.3.10 Reproductive assessment of adult oysters

Table 1–13. Data collected for each individual specimen to characterize reproductive status using classic histological methods.

Parameter	Unit
Sex	Male, female, simultaneous hermaphrodite (categorical)
Reproductive condition	Gametogenesis scale: 0–5 (categorical) (Gregory et al., 2023)

1.4 Quality Assurance Objective Criteria

The Data Quality Assurance Objectives (DQAOs) define a tolerable level of potential decision error for data collected on this project. They help to define the DQOs and clarify the project objectives further. The DQAOs are then used as comparison criteria during data quality review by FOB and AU to determine whether the minimum requirements have been met and the data may be used as planned. The quality assurance objectives are listed in **Tables 1–14 to 1–19**.

Unless explicitly stated, the same methods and metrics will be used for data collection at both intertidal and also subtidal oyster bed sites. Standardizing methods and metrics across sites will best enable comparison across sites.

1.4.1 Water-Quality

A data sonde will be deployed subtidally at each oyster bed site from March-November, and a HOBO temperature logger will be deployed subtidally at each site year-round. Each data sonde's DO, pH, conductivity, and chlorophyll-a sensor will be calibrated on a regular basis according to the manufacturers' instructions (see **Section 5**). The data sonde and HOBO temperature sensors cannot be calibrated, but they will be checked for drift and accuracy at least twice per year (see methods outlined in **Section 5**). Temperature data from each site will also be cross-validated by comparing concurrent data sonde versus HOBO data from the same site. Data sondes will be removed from oyster bed sites during the off season months (Dec–Feb) to avoid damage or loss due to storms or ice scour in shallow waters.

Table 1–14. DQAOs for collected water quality data.

Parameter	Method	Precision	Accuracy	Completeness
Water Temperature	HOBO Water Temp Pro v2	0.02°C	0.2°C	90%
Water Temperature	Aqua TROLL 600 sonde (In Situ, Inc); EPA 170.1	0.01°C	0.1°C	90%
Salinity	Aqua TROLL 600 sonde (In Situ, Inc); EPA Std. Methods 2520A	0.1	1	90%
Dissolved Oxygen	Aqua TROLL 600 sonde (In Situ, Inc); EPA-approved In-Situ Methods (under the Alternate Test Procedure process): 1002-8-2009, 1003-8-2009, 1004-8-2009	0.01 mg/L	±0.1 mg/L from 0 to 8 mg/L; ±2% of reading from 20 to 50 mg/L	90%
pH	Aqua TROLL 600 sonde (In Situ, Inc); EPA	0.01	0.1	90%

	Std. Methods 4500-H+, EPA 150.2			
Chlorophyll a	Aqua TROLL 600 sonde (In Situ, Inc)	0.001 RFU	0.01 RFU	90%

1.4.2 Oyster Bed Characteristics

Table 1–15. DQAOs for collected oyster bed characteristic data.

Parameter	Method	Precision	Accuracy	Completeness
Latitude, longitude	GPS	4 m	2 m	100%
Oyster bed length, width	Measuring tape	1 m	1 m	90%
Oyster bed area of coverage	Measuring tape	± 10% of true value	± 10% of true value	90%
Oyster bed tidal elevation (intertidal sites)	USGS tide gauge reference	10 cm	10 cm	90%
Oyster bed maximum and minimum depths (subtidal sites)	Depth gauge (SCUBA)	1 m	1 m	90%
Contoured chain length (for rugosity)	1 meter ball chain	0.5 cm	0.5 cm	90%
Straight-line distance (for rugosity)	Measuring tape	0.5 cm	0.5 cm	90%
Rugosity index	$\frac{Contoured\ chain\ length}{Straight-line\ distance}$	0.01	± 0.01	90%

1.4.3 Population demographics

Table 1–16. DQAOs for data collected on population demographics.

Parameter	Method	Sensitivity	Precision	Accuracy	Completeness
Oyster density	Counts in m^2 quadrats	1 oyster	PSE < 20%	±10%	90%

Oyster size distribution	Direct measurement	0.1 mm	PSE < 20%	±10%	90%

* PSE = Percent Standard Error (Described in **Section 4.1.1**)

1.4.4 Oyster recruitment, juvenile survival and growth

Table 1–17. DQAOs for collected recruitment, survival, and growth data.

Parameter	Method	Sensitivity	Precision	Accuracy	Completeness
Juvenile recruitment	Shell stringers	1 juvenile oyster	PE < 10%	Duplicate counts of settled spat on one shell per string will be recorded.	90%
% Survival	Shell bag counts	1 oyster	PE < 10%	Each replicate measured twice and third count if PE > 10%	90%
Growth	Direct measurement	0.1 mm	PE < 10%	10-20 juveniles will be measured twice each month**	90%

* PSE = Percent Standard Error (Described in **Section 4.1.1**)

* PE = Percent Error (Defined in **Section 4.1.1**)

** Minimum number is dependent on the number of surviving juveniles.

1.4.5 Disease Diagnostics

Table 1–18. DQAOs for collected disease diagnostic data.

Parameter	Method	Sensitivity	Precision	Accuracy	Completeness
P. marinus prevalence	RFTM	Limit of detection: 1,000 cells per g tissue (Bushek, 1994)	<10% RPD between multiple readers	A subset of slides (10-15) will be scored by multiple reviewers for	90%

				agreeance	
P. marinus intensity	RFTM	1 cell per sample	<10% RPD between multiple readers	Follow standard scoring criteria (modified Mackin scale); A subset of slides (10-15) will be scored by multiple reviewers for agreeance	90%
P. marinus, H. nelsoni, H. costale prevalence	qPCR (Piesz et al., 2022)	10 copies per sample	≤2.0% C_T between technical replicates	no template control	90%
P. marinus, H. nelsoni, H. costale intensity	qPCR (Piesz et al., 2022)	10 copies per sample	≤2.0% C_T between technical replicates	$10–10^8$ copies standard curve	90%
*(H. nelsoni & H. costale)*** prevalence	Histology	Sensitivity is low for low level infections and moderate for moderate-high infections. Species specificity is poor**	<10% RPD between multiple readers	A subset of slides (10-15) will be scored by multiple reviewers for agreeance	90%
*(H. nelsoni & H. costale)*** intensity	Histology	Sensitivity is low for low level infections and moderate for moderate-high infections. Species specificity is	<10% RPD between multiple readers	Follow standard scoring criteria (Carnegie & Burreson 2011); A subset of slides (10-15) will	90%

		poor**		be scored by multiple reviewers for agreeance	

* RPD = Relative Percent Difference

** **H. nelsoni and H. costale* are indistinguishable via histology, so both Haplosporidium spp. are included in reported metrics.

1.4.6 Biometrics and reproductive condition

Table 1–19. DQAOs for collected biometric and reproductive condition data.

Parameter	Method	Precision	Accuracy	Completeness
Shell measurements (height length, width)	Direct measurement	0.1 mm	Refer to standard	90%
Tissue condition	Visual Inspection (Howard et al. 2004)	<10% RPD between multiple readers	Follow standard scoring criteria	90%
Sex	Histology	<10% RPD between multiple readers	A subset of slides (10-15) will be scored by multiple reviewers for agreeance	90%
Gametogenesis index (1-5)	Histology	<10% RPD between multiple readers	Standard scoring criteria, and a subset of slides (10-15) will be scored by multiple reviewers for concordance	90%

* RPD = Relative Percent Difference

1.5 Special Training/Certifications

All personnel will receive training from subject matter experts and project leads Meghana Parikh and Katherine McFarland prior to data collection activities. In the event that boats are required to access sites, all core team members (Parikh, McFarland, Arnott, Kachmar) will receive NOAA vessel training, and on site NOAA Corps Officer will give final approval. All team members who participate in SCUBA diving on subtidal oyster beds will receive NOAA diver training and

certifications prior to completing these activities. The on-site NOAA Dive Coordinator will give final approval. All records and certificates will be archived by the NOAA approving agent, and stored on the project shared drive. If additional training needs arise, they will be considered and organized on a case by case basis to ensure all team members are properly trained to conduct field and laboratory tasks safely, while also meeting data quality objectives outlined in **Section 1.3**.

1.6 Documentation and Records

1.6.1 Documentation and Records

Field data will be collected using waterproof field notebooks/data sheets and digital photographs will be taken of field locations. Sonde records and digital caliper measurements will be downloaded to a digital tablet, either in the field or on immediate return to the laboratory. Laboratory data will be collected using lab notebooks, digital output logs from equipment, and in spreadsheets. Electronic data entry will be performed by a team member and checked by a second team member after entry. In addition, numerical data will be graphed and the resulting plots will be visually inspected to check for obvious outliers that need to be verified or corrected, as necessary. Data will be collated in a relational database.

1.6.2 Data Storage and Sharing

Data will be transferred from paper sheets and the local drive of a designated field laptop to a backed up location within 3 business days of collection. Collected data will be stored on a shared network drive within the NEFSC secure server system. Copies of .csv, .xlsx, .hobo, and other files generated, and output from utilized software, will be stored on the shared network drive. Field sheets will be digitally scanned or photographed, and their digital versions will be stored on the NEFSC shared network drive. All collected data, including the aforementioned software generated data, will be input into a data management system that is specific to this study and located on the NEFSC secure server system. All digital data storage locations are access-restricted to full-time NEFSC project personnel only, and all paper files will be stored in a designated filing cabinet in the office of Steve Arnott and Mariah Kachmar at the Milford Lab. Mariah Kachmar will be responsible for entering and securely storing all laboratory data collected on individual oyster samples (RFTM, qPCR, and disease and reproductive histology results). Steve Arnott will be responsible for entering and securely storing all field data (field sheets, field photographs, sonde recordings, field recordings). Meghana Parikh and Katherine McFarland will serve as administrators of the digital data management systems and have oversight over data storage and sharing on all servers and devices.

Following publication of results in peer-reviewed literature, clean, original field and laboratory data will be made publicly available through a GitHub (Github.com) Repository specific to this project (here) and published as a static repository using Zenodo to create a doi for the data archive and code for each manuscript produced. Metadata describing all collected data elements will be publicly available on the GitHub project page within one year of the initial QAPP approval and prior to publication to allow for transparency and individual data share requests.

After all data collection and associated quality assurance reviews for this project are complete, continuous water quality monitoring data, as well as biological field and laboratory data (if deemed appropriate), will be also uploaded to the EPA's WQX System for public archiving.

1.6.3 Permits

We have, or are in the process of obtaining, all necessary permits to collect oysters for scientific research. These include:

1. **Connecticut Department of Energy & Environmental Protection** - Permit to collect fish, crustaceans, and aquatic organisms for scientific & educational purposes.
   Permit number: SC-23001b
   Issued to: NOAA Fisheries
   Expires: January 30, 2026

2. **Connecticut Department of Agriculture, Bureau of Aquaculture** - Scientific Resource Assessment Permit
   In progress, and in direct communication with the permit-issuing office

3. **New York State Department of Environmental Conservation** - License to Collect or Possess: Marine Resources
   In progress

Additionally, we are coordinating with local organizations and officials (described in Sections 1.1 & 1.2.3) to provide awareness of and secure community support for the work outlined in this QAPP.

2. DATA ACQUISITION

Information on study site selection can be found in **Section 1.2.4** and **Table 1–4**.

2.1 Sampling Methods

2.1.1 Oyster bed water quality (field)

Aqua TROLL 600 data sondes (In-Situ, Inc.) will be deployed at each site for continuous monitoring of site specific water quality data. Parameters collected will include temperature, salinity, pH, dissolved oxygen, and chlorophyll a. Each sonde will be deployed in a protective PVC casing secured to a nearby permanent structure (e.g., a nearby dock; **Figure 2–1**) or fixed post. During winter months, when ice scour is a threat, the Aqua TROLL 600 sondes will be removed from each site and brought to the lab for maintenance and calibration before being deployed the following year. At least one HOBO Water Temperature Pro v2 data logger will be deployed throughout the year at each site to ensure year-round high resolution temperature data collection, and for cross-validation of temperature data from the Aqua TROLL 600 sonde. Each water quality parameter will be recorded once every 15 minutes.

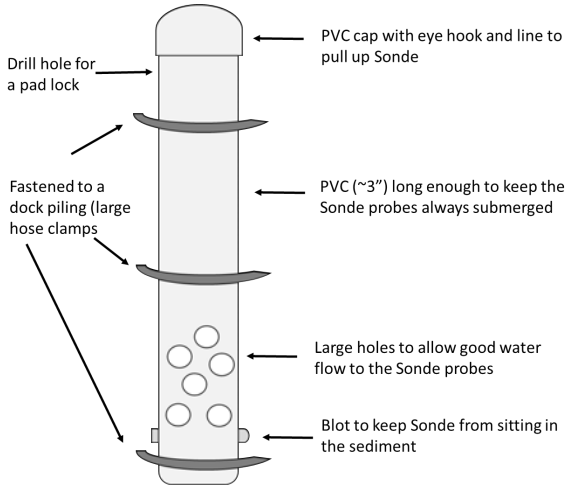


Figure 2–1. Schematic of the protective PVC housing that will be used to safely and securely deploy data sondes on derby dock pilings.

2.1.2 Assessment of oyster bed characteristics (field)

Sampling sites will be evaluated for total area of oyster bed coverage, oyster bed height above surrounding substrate, and either tidal elevation (intertidal sites) or depth (subtidal sites). They will also be assessed for rugosity (surface complexity), which is an important element of oyster bed health that affects processes such as productivity, accretion (Colden et al. 2017), water flow dynamics (Reidenbach et al. 2010), larval recruitment (Soniat et al. 2004), and trophic

interactions (Frost et al., 2005, Grabowski et al. 2008). The assessments will occur during May 2023 and May 2024.

Intertidal oyster beds

To determine area of oyster bed coverage, methods described in Janiak (2021) will be used. A measuring line will be placed along the longest axis of the oyster bed, with the perimeter defined as the continuous edge where live or dead shell make up ~25% of the substrate (Janiak, 2021). The total length of the oyster bed will be recorded, and markers will be placed at intervals along the measuring tape so as to divide the bed into five sections of equal length. The width of the bed will then be measured across each marker, perpendicular to the axis of the bed length. Area of the oyster bed will be calculated from the resulting polygon..

To identify areas of intertidal oyster beds that are at or below mean low water (≤ MLW, the zone from which oysters will be sampled for reproductive condition and diseases), sites will be visited at the expected time of MLW, based on NOAA tide predictions. Notes of clearly discernible landmarks will be taken, with reference photos, so that areas of oyster bed ≤ MLW can be easily identified for oyster sampling purposes.

To quantify oyster bed rugosity, measurements will be taken from at least ten randomly selected locations on the oyster bed. At each location, a fine-link chain will be contoured to the surface of the oyster bed along a straight line (**Figure 2–2**). (The final chain-link size and length will be determined after visiting the beds and testing multiple options. The decision will be made based on the bed's architecture, availability, and feasibility of standardized implementation all four sites). The straight-line distance between the beginning and end points of the chain will be measured to the nearest millimeter using a measuring tape, and an index of rugosity will be calculated as:

$$Rugosity = \frac{Contoured\ chain\ length}{Straight{-}line\ distance}$$

such that progressively more structured and complex surfaces have values that are increasingly greater than 1.

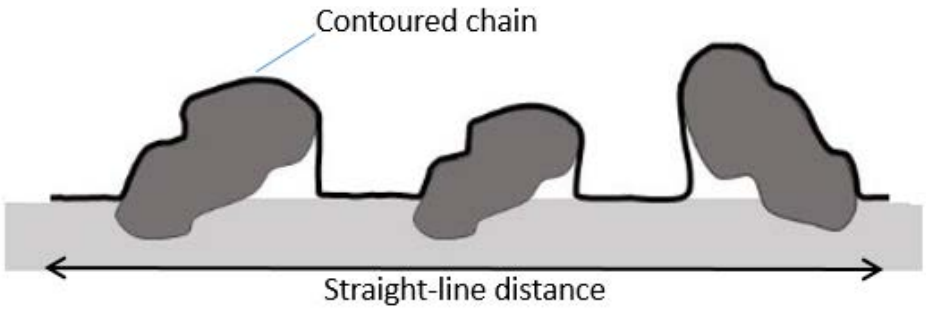


Figure 2–2. Schematic showing method of measuring oyster bed rugosity. A fine-link chain will be conformed to the surface of the oyster bed without pushing the chain into overhang crevices. Rugosity will be calculated as chain length divided by straight-line distance (Janiak, 2021).

Subtidal oyster beds

Subtidal oyster bed sites will be evaluated by SCUBA divers. To determine area of oyster bed coverage, methods described in Janiak (2021) will be used. A weighted measuring line will be placed along the longest axis of the oyster bed and secured to the seabed at each end. The total length of the oyster bed will be noted on an underwater slate, and markers will be placed at intervals along the measuring tape so as to divide the bed into five sections of equal length. The width of the bed will then be measured across each marker, perpendicular to the axis of the bed length. Area of the oyster bed will be calculated from the resulting polygon.
Oyster bed depth will be measured to the nearest 0.5 meter using two divers' depth gauges (one from each diving buddy pair). The gauges will be placed on the seabed at the deepest and shallowest points to determine minimum and maximum depths. Time of depth recordings will be noted. The time and depth data will be referenced against the nearest USGS tide gauge so that depths can be expressed with respect to mean low water.
Rugosity (surface complexity) will be quantified as described for intertidal oyster beds (above).

2.1.3 Population demographics (cover, density, mortality, and sizes) (field)

Surveys of oyster populations will be performed at each site near the beginning (April/May) and end (October) of the 2023 and 2024 field seasons. This will allow demographic parameters to be determined before and after the expected peak onset of disease (July-September) (Volety et al. 2000).

Quadrat sampling will be used to quantify demographic variables, such as total population size structure and density. All articulated oysters (those with hinged valves) will be counted, including adults (shell height ≥ 40 mm, or approximately 1 year and older), recently settled spat (<10mm), and all juveniles (shell height ≥ 10mm and < 40mm). Articulated oysters will be categorized as either live, gaping, or box, as defined in **Table 2–1**. The gaping and box categories will be used for calculating indices of adult mortality.

Samples will be taken from randomly placed quadrats. A minimum of three quadrat samples will be taken per site and sampling event, although the final number of quadrat samples taken, and the size of quadrat used (either 25 x 25 cm or 50 x 50 cm) will be determined based on precision and efficiency targets, as described in **Section 1.4.3**. Once a quadrat has been placed, the percent of area covered by articulated oysters (live, gapers and box combined) will be visually categorized into one of five categories (<20%, 20-40, 40-60, 60-80, >80% coverage) and the enclosed area will be photographed with a digital camera. Articulated oysters will then be excavated from quadrats and enumerated by oyster category (**Table 2–1**). Up to 30 individuals of each category will be measured using calipers to the nearest 0.1 millimeter for shell height (but not shell length or depth, which are difficult to determine in the field for

individuals attached in clumps), as shown in **Figure 2–3**. Mortality indices (adapted from Ford et al., 2006) will be calculated as follows:

$$Total\ mortality = \frac{number\ of\ gapers + box\ /\ m^2}{number\ of\ gapers + box + live\ oysters\ /\ m^2}$$

$$Gaper\ mortality = \frac{number\ of\ gapers\ /\ m^2}{number\ of\ gapers + live\ oysters\ /\ m^2}$$

Percent cover, density counts, and shell measurements will be performed *in situ*. Specimens will be returned immediately to their original location in order to preserve integrity of the bed, except for a sub-sample retained for reproductive and disease assessments (see below).

Table 2–1. Description of oyster categories that will be assessed for population demographics. Source: Tarnowski, 2021.

Oyster category	Description
Live	Alive oyster
Gaper	Dead or moribund oyster with gaping valves and tissue still present.
Box	Pairs of empty shells joined together by their hinge ligaments. These remain connected for months after the death of an oyster, providing a durable estimator of recent oyster mortality. Recent boxes are those with no or little fouling or sedimentation inside the shells, generally considered to have died more recently (weeks or a few months). Old boxes have heavier fouling or sedimentation inside the shells and the hinge ligament is generally weaker. Time until valve separation depends on size, salinity, and temperature (Ford et al., 2006).

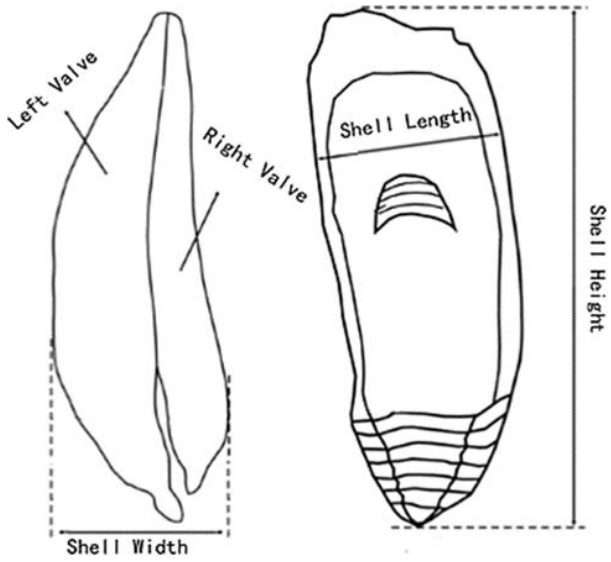


Figure 2–3. Oyster shell height will be measured in the field. Shell height, length, and width will be measured in lab specimens.

2.1.4 Recruitment of oyster spat (field)

Oyster recruitment will be quantified at each site during 2023 and 2024 using oyster shell stringers (**Figure 2–4**). Each shell stringer will consist of 12 dead oyster shells (shell heights, 5-10 cm) oriented inner (concave) surface down with holes drilled through the center and strung together using weighted galvanized wire (Haven & Fritz, 1985; McFarland et al., 2022). Prior to deployment, shells will be soaked in bleach for 48 hours, scrubbed clean with a wire brush, then soaked in freshwater for at least 24 hours (Knoell et al., 2021).

Based on previous studies (Loosanoff, 1966), spatfall in LIS is expected from July through October. Therefore, shell stringers will be deployed twice per year, first in June and again in August. A minimum of three shell strings (12 shells each, 36 shells total) will be deployed per oyster bed site (below mean low water if intertidal) on each deployment date, with strings suspended ~5 cm above the seabed on PVC frames. Shell stringers will be assessed for recruitment after two months (August for the June-deployed bags; October for the August-deployed bags). At the time when recruitment is assessed, shell stringers will be returned to the laboratory so that oyster spat can be verified under a dissection microscope. Recruitment will be quantified as the mean number of newly settled oysters per oyster shell, deployment date, and site.

Figure 2–4. Underwater photograph of oyster shell stringers suspended from a PVC T-bar frame, as used by McFarland et al. (2022).

2.1.5 Growth and survival of juvenile oysters (field)

During peak oyster spat settlement in year 1 (Aug-Sep 2023), shell bags will be deployed at each site to collect new spat. These will serve as a source of juvenile oysters for growth and survival experiments. Shell bags (~30 cm long, ~15 cm filled diameter) will consist of polypropylene mesh (~15 mm^2 openings) filled with whole, unarticulated shells from dead oysters to serve as a substrate for spat settlement (McFarland and Hare, 2018; **Figure 2–5**).

In October 2023, after peak settlement, shells will be removed from the bags and examined for spat. Shells with settled spat will be chosen for growth and survival experiments. Each selected shell will be uniquely labeled, its number of attached spat will be enumerated, and spat shell heights will be measured to the nearest 0.01 mm using calipers (either all spat per shell, or a sub-sample of 15 spat per shell if densities are greater than 15 spat per shell). Labeled shells will be distributed equally among three cages (1 m x 0.5 m x 0.5 m high enclosed in 25 mm wire mesh) to exclude large predators, with the number of shells being dependent on spat density (goal of 50–100 spat per cage). Cages will be secured to the seabed using weights and/or metal stakes adjacent to the oyster bed where the spat were collected (below mean low water for intertidal sites). The exact method of securing cages will be determined based on the substrate, depth, and tidal flow at each specific deployment site. Spat will not be moved among sites.

From April-October 2024, monthly counts of the caged juvenile oysters (those that settled during Aug-Sep 2023) will be recorded to monitor survival over time. Each replicate of juvenile oysters will be counted twice to assure accuracy. If the counts are within a margin of <10% the two counts will be averaged. If the margin of error is >10%, a third count will be conducted and the two closest values will be averaged, providing they are within a margin of <10% (otherwise, additional counts will be taken until consistency is achieved). Shell height will be measured from 50-100 randomly selected individuals per month (with replacement, as above) to monitor mean growth rates. Any new spat that settle inside the cages during 2024 (identified as those with a

shell height less than the previous month's minimum) will be removed and discounted from analyses.

To ensure quality control of survival counts, the percent error method will be implemented, as described in **Section 4.1.1** (precision of field data). To ensure quality control of the growth data, up to 20 juveniles will be measured twice each month, first by a primary measurer, and then by a secondary measurer. A paired t-test will be performed on these data using α = 0.05 to ensure that there is no systematic bias between readers, and the percent error method will be used to quantify the level of agreement.

Figure 2–5. Shell bags used by McFarland and Hare (2018) to collect spat for growth experiments. Shell bags were made of polypropylene mesh (~15 mm^2 openings), and measured ~30 cm long by ~15 cm diameter after being filled with dead oyster shells.

2.1.6 Collection of adult oysters for disease and reproductive assessments (field)

A total of 30 oysters from each site will be collected monthly from April to November 2023 and March to November 2024 to assess pathogens, disease burden, and reproductive condition. Each oyster will be selected from random, spatially separated locations across the bed (not as clumps) to ensure that they are statistically independent of one another for the purposes of data analysis. To minimize disruption to oyster beds during times of the year when demographic studies are being performed (April/May and October - see above), a portion of the kept specimens will come from the quadrat (demographic) samples. However, only 1-3 oysters will be kept per quadrat to ensure spatial coverage and statistical independence (oysters not kept from quadrat samples will be returned to the oyster bed). All specimens kept for lab work from intertidal sites will be collected from the lower tidal zone of the oyster bed (the subtidal portion of the oyster bed, below the mean low water mark). If oyster bed density or availability of suitable oyster sizes prohibits the targeted sample size of n = 30 per month, we will adjust the sample size to a minimum of 15 oysters per month, with input from local shellfish groups. Adult oysters with a shell height ≥ 40 mm will be targeted to ensure sexual maturity (Harding et al. 2013), although this may vary depending on mean sizes available at different sites. Shell lengths ≥ 40 mm will also be targeted because the diseases of interest tend to have the greatest effects on large oysters aged 2 years and older (Volety et al. 2000).

Once collected, live oysters will be immediately stored on ice in labeled bags and returned to the laboratory for disease and reproductive analyses. Each individual will be used for both disease tissue sampling and histological assessment of reproductive condition.

2.1.7 Preparing oysters for tissue sampling (lab)

Full methods are described in **Appendix A**. Upon arrival at the Milford Laboratory, oysters will be processed immediately, or refrigerated at 4℃ if time is limited. All oysters will be processed to the point of tissue preservation within 48 hours of field collection, with the goal of completing sooner. Oysters will be scrubbed free of sediment and biofouling and undergo a cold freshwater rinse. Measured biometrics will include shell height, length, and width to the nearest 0.1 millimeter (**Figure 2–3**), and total wet weight to the nearest 0.1 gram. Once shucked, oysters will be assigned a body condition rating of fat, medium, or watery to indicate overall health of the individual (Howard et al., 2004).

2.1.8 Sampling oyster tissues for pathogen detection and reproductive assays (lab)

Individual and population-level pathogen prevalence and intensity will be evaluated using three diagnostic assays. Tissue samples for all diagnostics will be collected from each oyster, maximizing the sample size for each diagnostic test and minimizing the number of animals sacrificed. Gill, digestive diverticula and rectal tissue will be used to quantify the burdens of *P. marinus*, *H. nelsoni*, and *H. costale* using a novel triplex qPCR assay (Piesz et al. 2022). This method has been used by Roger Williams University, Aquatic Diagnostic Laboratory (RWU ADL) for surveillance of Connecticut's cultured oysters since 2019 (State of Connecticut Department of Agriculture, 2022). Modern molecular diagnostics can provide an advantage over traditional histopathology and culture techniques because of increased assay sensitivity and high-throughput processes.

Results from the novel triplex qPCR assay will be compared against results from traditional cultures with Rays Fluid Thioglycollate Medium (RFTM) and histopathology to validate concordance among assays. A 5 x 5 mm section of mantle tissue will be collected for the RFTM assay of *Perkinsus spp.* burden. This will be followed by a cross-section for histology (including portions of the mantle, digestive tract, gonads, kidney, and gills), which will be fixed in Davidson's fixative (Fisher et al., 1996) and washed in ethanol. Histological sections will be used to identify infection by *Perkinsus* and *Haplosporidium spp*., and to evaluate reproductive stage **(Appendix A).**

Table 2–2. Description of adult oyster tissues to be sampled from adult oyster specimens

Approach	Target	Tissues	Size of Tissues	Preservation
Molecular (Triplex qPCR)	*P. marinus* *P. nelsoni* *H. costale*	Gill, gut, and rectal tissue	0.5 mg (combined)	95% ethanol
Microscopy	*P. marinus*	Mantle adjacent to the palps	5x5 mm section	Ray's fluid thioglycollate medium

				(RFTM), 5-7 days
Histology	*P. marinus* *P. nelsoni* *H. costale* Tissue condition	Transverse section of tissue (~4 mm) that includes the mantle, digestive tract, gonads, kidney, and gills	~4mm thick whole body cross section	Davidson's fixative (24 hrs), then 70% ethanol
Histology	Sex Gametogenesis index	Same tissue sample as for parasite histology	~4mm thick whole body cross section	Davidson's fixative (24 hrs), then 70% ethanol

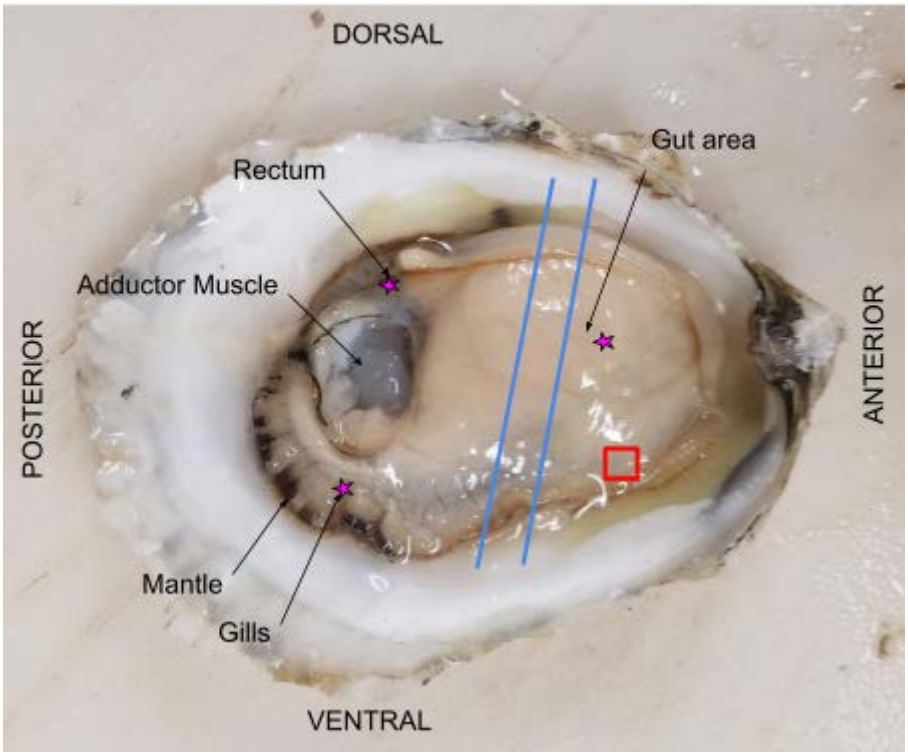


Figure 2–6. *Crassostrea virginica* anatomy showing tissues that will be sampled for disease diagnostics and reproductive conditioning. Tissue between the blue parallel lines will be sampled for histology (includes mantle, digestive tract, gonads, kidney, and gills). The red square shows mantle tissue to be sampled for RFTM preservation. Pink stars show locations of tissues that will be sampled for triplex qPCR (gill, gut, and rectal tissues).

2.1.9 Archive tissue for future research

During 1–3 months of sampling in year 1, tissue processing will include the preservation of additional tissue samples (from the same oysters) for potential future population genetic assessments. From all oysters dissected for disease and reproductive assays, a small piece of adductor muscle (~ 5 mm^3) will be dissected and preserved in 95% Ethanol (see **Appendix A** for detailed SOP). Creation of this archive, in collaboration with efforts by the U.S. Department of Agriculture and Cornell University to archive oyster tissue, will allow for an understanding of how population genetics on oyster beds change over time as a result of climate change and / or restoration practices (Hornick and Plough, 2019, 2021). Molecular analysis of samples for genomics is outside the scope of the work outlined in the QAPP. However the opportunistic sampling of adductor muscle tissue from the present work maximizes the potential scientific contributions from the sacrificed oysters and reduces the need for destructive sampling in the future. Adductor muscle tissue preserved in 95% ethanol and frozen at -80°C can be stored indefinitely. Any future analysis of the archived tissue will be performed under the provisions set forth in a separate QAPP developed for this purpose.

Laboratory analytical methods for detection of pathogens and reproductive scoring are detailed in **Section 3** (Analytical Requirements).

2.2 Sampling Information

Details of the sampling locations can be found in **Table 1–4**. Timing of the monthly field visits will coincide with low tide for intertidal oyster beds for ease of access and improved ability to work. All field site visits will be planned within a flexible window to accommodate inclement weather that would limit our ability to collect quality data and/or put team members in danger. Detailed methods for all field and laboratory data collection are outlined in **Section 2.1**, with detailed SOPs included as appendices (**Appendix A**) of this document.

2.2.1 Field Documentation

All field data will be documented in a waterproof field notebook on pre-printed waterproof data sheets. Field sheets will be digitally backed up in the field, and/or on return to the laboratory (**Section 1.6.2**). Details of data collections for each field parameter are described in **Section 1.3**.

2.2.2 Field Instrument Calibration

Field instruments (AquaTroll 600 data sondes, HOBO data loggers, and calipers) will be calibrated regularly as described in **Section 5** of this QAPP to ensure precision and accuracy of data collected.

2.2.3 Decontamination Procedures

All equipment will be thoroughly rinsed with freshwater after use and between field sites. At the end of field days, all gear will be left to air dry after the freshwater rinse. In the lab, all laboratory tools will be decontaminated between individual oysters using alcohol and flame sterilization (detailed in **Appendix A**). In molecular lab spaces, workbenches will be sanitized between

sample batches using 70% ethanol. Oyster tissue processing areas will be disinfected after use using either 4.25% hydrogen peroxide, or a 1:10 dilution of 6% sodium hypochlorite.

2.3 Sample Handling Procedures

Oysters collected from the field will be handled according to methods described in **Section 2.1.8** of this QAPP, and in accordance with the chain of custody detailed below.

Sample containers for laboratory tissue storage and analysis will either be cleaned between samples, or freshly obtained as required for the specific assay. Details of appropriate sample containers and storage requirements are given in the laboratory SOPs (**Appendix A**) as well as in **Table 2–3**.

Table 2–3: Sampling collection and container requirements

Sample	Sample Container	Required Cleaning/ Free-from Certification	Typical Sample Volume	Preferred / Maximum Holding Times
Triplex qPCR - Gill, gut, rectal tissue	Microcentrifuge tube	DNAse/RNAse free	0.5mg tissue in 1.5mL tube	Hold indefinitely preserved in 95% ethanol
Tissue archive - adductor muscle	Microcentrifuge tube	DNAse/RNAse free	1.5mL tube	Hold indefinitely preserved in 95% ethanol
RFTM culture - mantle tissue	Glass culture tube with screw cap	Autoclave tubes and culture media for 15 minutes at 121℃ (15psi)	5 x 5 mm tissue in 15mL RFTM	Preferred 5–7 days in the dark at room temperature, or up to 3 months at 4℃.
Histology sections - oyster cross section	Histology cassette; high density polypropylene histology containers with polyethylene screw cap.	Non-sterile; single use histology cassettes. Wash histology containers with laboratory detergent and rinse with DI water between uses.	15:1 - 20:1 ratio fixative to tissue volume. Tissue cross section thickness ~4 mm.	Preferred 24 hrs in Davidson's fixative, followed by preservation in 70% ethanol < 30 days.

2.3.1 Sample Identification

All tissue samples will be labeled with the following information:

1. Collection site
2. Collection date (MM/DD/YYYY)
3. Tissue type(s)

4. Unique oyster ID number that corresponds to all other measurements logged for this individual

Additionally, all tissue samples will be logged in a master file that contains all sample information (listed above), its storage location, and the status of downstream analysis (eg. histology, RFTM, qPCR).

2.3.2 Chain of custody and sample transfer field collection to lab

All oysters will be collected by hand and stored on ice in coolers for transport from the field site to the Milford Laboratory. Oysters will be stored in the refrigerator overnight or until processing can be completed (no more than 48 hours). Oysters will be processed at the Milford Laboratory and excess tissue and shell will be discarded according to Northeast Fisheries Science Center, Milford Laboratory waste management protocols. Tissue will be frozen and discarded in a dumpster on garbage pick up day. Shells will be stored in buckets in a walk-in refrigerator and either disposed of with the tissue samples, or donated to local CT shell recycling programs, if appropriate.

3. ANALYTICAL REQUIREMENTS

Field sampling and tissue processing methods are described in detail in **Section 2.1**. Methods for laboratory analyses of collected samples are detailed in SOPs (**Appendix B–G**) and below.

3.1 Analytical Methods

3.1.1 Disease Diagnostics: Triplex qPCR assay for pathogen detection (lab)

Presence and abundance of *P. marinus, H. nelsoni*, and *H. costale* DNA will be assessed by extracting total DNA from oyster gill, digestive diverticula, and rectal tissue, then conducting species-specific qPCR assays using methods described in Arnishi and Okimoto (2006), and Piesz et al. (2022) (**Appendix C & D**). Intensity of infection will be reported based on the DNA copy number within each tissue sample. For *P. marinus*, DNA copy numbers have been correlated with the modified Mackin scale (Mackin, 1962) from 0 to 5, with 0 representing individuals with no infection and 5 representing individuals heavily infected (Roger Williams University, Aquatic Diagnostic Laboratory, *unpublished data*). Intensity of *H. nelsoni* and *H. costale* infections will be reported from 0 to 3, with 0 representing no infection and 3 representing individuals that are heavily infected. These scores are based on DNA copy number ranges established by RWU, ADL (*unpublished data*).

For all diagnostic modalities, population level metrics of infection prevalence and intensity will be calculated as follows (also described in **Sections 1.3.7 – 1.3.9**).

$$Prevalence = \frac{number\ of\ infected\ oysters}{total\ number\ of\ oysters\ in\ the\ sample}$$

$$Intensity\ (population) = \frac{sum\ of\ individual\ intensity\ scores}{number\ of\ infected\ oysters\ in\ the\ sample}$$

$$Weighted\ Prevalence = \frac{sum\ of\ individual\ intensity\ scores}{Total\ number\ of\ oysters\ in\ the\ sample}$$

3.1.2 Disease Diagnostics: Ray's Fluid Thioglycollate Medium assay for detection of *P. marinus* (lab)

Presence and abundance of *P. marinus* cells will be assessed using sections of mantle that have been excised from just above the palps and incubated in Ray's fluid thioglycollate medium for 5–7 days (Ray, 1954). After incubation, tissue sections will be macerated on a microscopy slide, stained with Lugol's iodine, and analyzed microscopically. Intensity will be scored on a scale based on parasite cell density within the tissue sample using a modified Mackin scale (Mackin, 1962) from 0 to 5, with 0 representing individuals with no infection and 5 representing individuals heavily infected (**Appendix B**). Population level metrics of infection prevalence and intensity will be derived as described in **Section 3.1.1**.

3.1.3 Disease Diagnostics: Histology for pathogen detection and diagnosis of disease (lab)

Cross-sections of oyster tissue previously fixed in Davidson's Fixative and preserved in 70% ethanol will be run through an automatic processor and embedded in wax blocks. Tissue sections will then be cut to 5 µm thickness on a microtome, mounted on slides, and stained with Harris' hematoxylin and eosin (Howard, 2004; **Appendix E**). Slides will be examined microscopically for presence of *P. marinus*, *H. nelsoni*, and *H. costale*, as well as tissue changes indicative of infection. *H. nelsoni and H. costale* are indistinguishable via histology, so both species are included in reported metrics. Intensity of *Haplosporidium spp.* will be scored on a scale based on parasite cell density and tissue localization from Carnegie & Burreson (2011) from 0–4, with 0 representing individuals with no infection and 4 representing individuals with heavy infection. Intensity of *P. marinus* will be scored on a semi-quantitative scale based on parasite cell density and tissue localization from Mann et al. (2014). Population level metrics of infection prevalence and intensity will be derived as described in **Section 3.1.1.**

3.1.4 Reproductive assessment of adult oysters (lab)

All procedures are detailed in **Appendix G**. Progression of reproductive maturity and spawning will be monitored monthly using standard histological techniques (National Research Council, 1980; Fisher et al., 1996). Histological sections used to evaluate pathogen burden will also be used to determine oyster gametogenic stage. Reproductive status will be ranked on a gametogenesis index (GI) scale of 0–5 (Gregory et al. 2023).

3.2 Laboratory Standards and Reagents

All media, reagents, antimicrobials, and standards used for laboratory analyses described in **Section 3.1** and associated SOPs will be regularly inventoried by primary laboratory personnel. Date of preparation, analyte or mixture, concentration, name of preparer, lot or cylinder number, and expiration date, if applicable, will be recorded on each working solution. Aseptic technique will be used whenever possible to prevent contamination of stock ingredients and working solutions. Details of preparations used during lab procedures will be recorded in a laboratory notebook so they can be tracked and discarded if results suggest that contamination may have occurred. Details regarding the appropriate storage and handling of laboratory standards and reagents are included in the specific SOPs pertaining to them.

4. QUALITY CONTROL REQUIREMENTS

4.1 Measurement Performance Criteria

The overall QA objective for this project is to develop and implement procedures for data collection and reporting that will provide results that are scientifically defensible. Specific procedures for reporting of data, internal QC, audits, preventive maintenance of field equipment, and corrective action are described in the other sections of this QAPP (see **Section 1.3** for data quality objectives). SOPs and the approved quality assurance plan (this document) will be printed and housed at the relevant workstations to ensure uniform execution of all procedures. All personnel will have access to finalized, digital copies of the same guidance documents.

4.1.1 Field Precision

At each site, mean oyster density will be quantified by placing quadrats in random locations across the oyster bed. The area enclosed by each quadrat will be photographed to create a visual record, and the percent surface area covered by whole articulated oysters will be quantified. Articulated oysters will be examined to determine whether they are live, gaping (moribund), or box (dead) (see **Table 2-1**), and the numbers of each type per quadrat will be enumerated. Oyster counts will be taken from a minimum of three quadrats, as recommended by Janiak (2021), but the final number will be determined by targeting a percent standard error (PSE) of less than 20%. PSE, which is a measure of precision (Whitlock and Schluter, 2019), will be calculated as:

$$PSE = 100 \times \frac{se}{\overline{d}}$$

where $\overline{d}$ represents the mean number per square meter and se is the associated standard error. If a target precision of PSE < 20% is not achieved using three quadrats, additional quadrat counts will be taken until the target level is achieved.

The size of the quadrat used for taking counts will be based on efficiency (time taken) to achieve the target level of precision (Krebs, 1999). Efficiency is important because the time available for field surveys will be limited by tide duration (intertidal sites), or safe and feasible dive times (subtidal sites). At the beginning of the study, two quadrat sizes will be assessed at each site: a smaller quadrat measuring 0.25 x 0.25 m (e.g., McFarland et al., 2022), and a larger quadrat measuring 0.5 x 0.5 m (e.g., Baggett et al., 2014). The final quadrat size may differ between sites, based on conditions (e.g. low density sites may require a larger quadrat than high density sites).

The shell heights of oysters (**Figure 2–3**) collected during quadrat counts will be measured to the nearest 0.1 millimeter using calipers. The mean shell height will be calculated for each quadrat. As with the oyster counts (above), we will target a PSE < 20%. After the initial sampling event, the calculated PSE will be considered, along with sampling efficiency (time taken) to determine the final number of quadrat replicates needed for estimating shell height.

When assessing recruitment of spat on shell stringers, at least one shell from each string will be recounted by a second observer (Knoell et al, 2021). The percent error (PE) between the two observers will be calculated as:

$$PE = 100 \times \frac{(count_1 - count_2)}{(count_1 - count_2) / 2}$$

where $count_1$ is the count from the primary observer and $count_2$ is the count from the secondary observer. If PE is greater than 10%, the primary counter will recount all shells, if necessary, after reconciling the source of discrepancy with the secondary observer.Multiple data readers and recorders will be used, and detailed SOPs will be followed by all. Additionally, regular calibration of field instruments will be performed.

Methods to ensure precision of spat survival counts and juvenile growth measurements are described in **Section 2.1.5**.

4.1.2 Laboratory Precision

Data quality assurance objectives for laboratory based procedures are outlined in **Sections 1.4.5** (Disease Diagnostics) and **1.4.6** (Biometrics and Reproductive Condition). Duplicate sample reads for histology and RFTM from a random subset of samples each month will be conducted to assure disease intensity and gonad development rankings are consistent across readers. If discrepancies arise, a third reader will participate and a full review of ranking systems will be reviewed by all team members. A relative percent difference ≥10% between reader scores will initiate additional duplicate slides to be read by the second reader to assure data quality.

Guidelines and landmarks for shell measurements and other steps in the process will be discussed among members prior to start to ensure uniformity. For tissue sectioning for histology, there will be a gauge at the station to ensure proper thickness of the cross section and photos will also be posted in the area for easy reference.

For the triplex qPCR disease quantification, duplicate reactions will be run for each sample to assure precision of the sample results. Technical replicates should have ≤2.0% difference in C_T reads. Failure to meet this DQAO will initiate a second qPCR run of the sample, but in triplicate.

4.1.3 Field Accuracy

Field accuracy of the water quality data will be controlled by calibrating sondes and HOBO loggers on a regular basis according to the manufacturer's suggestions. Calibration will include a comparison of sonde/HOBO measurements to standards for each parameter measured.

All team members will have hands-on training prior to data collection. Project Managers Parikh and McFarland will serve as subject matter experts in their respective fields for training purposes. These steps will ensure all members are capable of safely performing tasks they are responsible for on this project and that all data are collected in the same way among all team members.

During all site visits, details about weather events, extreme biofouling, and all unexpected occurrences will be recorded in the field notebook to add context and ensure data accuracy.

4.1.4 Laboratory Accuracy

Data quality assurance objectives for laboratory based procedures are outlined in **Sections 1.4.5** (Disease Diagnostics) and **1.4.6** (Biometrics and Reproductive Condition). Duplicate sample reads for histology and RFTM for a random subset of samples each month will be conducted to ensure disease intensity and gonad development rankings are consistent across readers. If discrepancies arise, a third reader will participate and a full review of ranking systems will be reviewed by all team members. A relative percent difference ≥10% between reader scores will initiate additional duplicate slides to be read by the second reader to ensure data quality.

Regular calibration and maintenance of all laboratory equipment will be conducted according to the manufacturers guidelines. All team members will have hands-on training prior to laboratory work and data collection. PIs Parikh and McFarland will serve as subject matter experts in their respective fields for training purposes. We will work closely with the CT DABA shellfish pathologist to ensure quality control of the slide preparation and reading for histological assessment of infections and reproductive condition.

DNA plasmid qPCR standards containing 10–10^8 copies of DNA for each of the three pathogens, *P. marinus*, *H. nelsoni*, and *H. costale* will be run with each plate to enable the accurate quantification of pathogen DNA present in each test sample. The triplex qPCR assay has been thoroughly tested for sensitivity and specificity. The assay has a detection limit of 10-100 copies of plasmid DNA for *P. marinus* and *Haplosporidium spp.* and 80-100% concordance between singleplex qPCR for *P.marinus* and *H. nelsoni*, and gel-based PCR for *H. costale* for detection. The overall concordance between histology and qPCR was 54, 57, and 87% for *P.marinus*, *H. nelsoni* and *H. costale*, respectively. Discordance is due to increased sensitivity of the qPCR assay compared to histology which can have false negatives (Piesz et al., 2022).

4.2 Internal Quality Control

Internal QC will be achieved during multiple steps of the project. First, detailed data sheet logs will be created to serve as a check that all data are obtained while in the field. A team lead will check that the data log is filled out to completion (all data points recorded) prior to departure from the field site. Once data are in hand QC will be achieved by transcribing data collected in the field (notebooks and data sheets) within 3 business days of the date of field collection. Digital data files will then be QC'd by a second project team member alongside the field data sheets and notebooks to assure all data was correctly entered within one week of data entry. Once QC'd, data will be collated with data collected in previous months for archiving and exploratory plots and summary statistics will be updated as a second check to ID problematic or outlier data. The QA managers will review the data monthly for completeness and accuracy, and will take measures to ensure updates to the data itself or the collection process are updated

prior to the next site visit. All QC steps will be logged with identification of the team members completing each QC step. Lastly, field data sheets will be scanned and the digital version backed up on the secure NOAA server and the physical original copy filed at the NOAA Milford Laboratory. These steps will ensure that the data quality control objectives and quality assurance objectives previously outlined (**Sections 1.3 and 1.4**) are met.

4.3 Field Quality Control

All team members will be trained to complete all field tasks, including training on data entry requirements for each specific task. To ensure completeness, data sheets will include a checklist of all data that needs to be recorded during each visit. Detailed standard operating procedures as described in **Section 2.1** will be compiled into a field manual to assure consistent methods of data collection by all. For water-quality parameters, the equipment calibration will serve as the QC to quantify precision and accuracy and to identify any limitations of the associated data.

4.4 Laboratory Quality Control

Specific laboratory quality control measures are outlined in individual SOPs. These may include having laboratory members wear gloves and lab coats to reduce the risk of contamination. All equipment will be sterilized before and after use, as well as in between individual organisms. Tubes and slides will be pre-labeled prior to beginning the assay to streamline the process and prevent samples from being incorrectly identified.

There will be designated stations/lab areas for each process. Upon arrival from the field, oyster samples will be rinsed and scrubbed clean as described in the Oyster Tissue Processing and Preservation SOP (**Appendix A**) in the NOAA Milford lab's Field Sample Processing Lab. Scrubbed oysters will be transported to a Biosafety Level 2 Certified (by CT Department of Public Health) lab space, where oysters will be opened and tissues processed and/or preserved for disease diagnostics by RFTM and qPCR. Preparation and preservation of samples for histology will be completed in a separate space fitted with a chemical fume hood. Fixed histology samples will be transported to the CT DABA shellfish pathology lab for processing. The Milford lab has a designated room for DNA extraction from ethanol preserved tissues, and a separate room to execute the PCR plate set-up and run the thermocycler to reduce potential contamination.

Lab members will use a "PCR hood" that is compatible with UV light to sterilize the area before preparing the assay. Only the assay will be prepared in the hood. No DNA or plasmid DNA is to enter the hood to reduce contamination in the assay. All hoods (i.e., chemical fume, PCR laminar flow, and biosafety cabinets) are inspected and certified annually to ensure safe and effective functioning.

An inventory log of all required chemicals, reagents, and supplies present in the described lab spaces will be maintained. Documented details will include the product name, manufacturer, Chemical Abstract Service (CAS) registry number, volume, quantity, expiration date, storage location, and storage requirements. The inventory will be reviewed at least quarterly to ensure the product quality and availability.

5. INSTRUMENTATION & EQUIPMENT PREVENTIVE MAINTENANCE

5.1 Sample Equipment Cleaning Procedures

As described in **Section 1.4.1**, sampling equipment will be cleaned after each use and between sites. Sondes and HOBO loggers will be cleaned monthly during their deployment. Sondes will be removed from sites during the winter months (December - February) to avoid damage or loss due to ice scour in our relatively shallow waters. During this time a thorough annual cleaning will be performed. Sondes will be fully dried, excluding probes that need to remain moist, O-rings replaced and lubed where required, and all systems checked for performance.

5.2 Instrument and Equipment Testing Procedures and Corrective Actions

5.2.1 Field Equipment

The DO, pH, chlorophyll-a, and conductivity sensors of the In-Situ Aqua TROLL 600 data sondes will be calibrated at the beginning of each field season, every 10–12 weeks during site visits (March - November), and factory calibration will occur annually in the winter. Thus, calibrations will occur well within calibration frequencies recommended by In-Situ. Prior to calibration, sensors will be tested against standards to check for instrument drift (as recommended on p. 46 of the Aqua TROLL 600 manual). If significant drift is observed during monthly data checks (as described in later text), instrument calibration will be completed sooner than the regularly scheduled interval.

Field calibration of the pH and conductivity sensors will be performed using the Quick Cal Solution method described on page 47 of the Aqua TROLL 600 manual.

In-Situ provides several methods for calibrating their DO sensor. We will use the water saturated air (damp sponge) method, described on page 41 of the Aqua TROLL 600 manual, because it is the most accurate method (In-Situ technical support, pers. comm. 4/10/2023), and easily performed in the field. Each DO sensor requires a cap in order to function properly. We will fit each DO sensor with an RDO-X cap (In-Situ part number 0079790), which should not need replacing during our two-year project (expected life of 24 months according to the In-Situ specification sheet). If any replacements are necessary, however, new RDO-X caps will be fitted according to instructions on the In-Situ RDO-X Cap Replacement Kit instruction sheet.

The chlorophyll-a sensor will be calibrated using the deionized water method specified on page 2 of the In-Situ chlorophyll-a sensor overview document, as recommended to us by the In-Situ technical support team (pers. comm. 4/10/2023).

It is not possible to calibrate the temperature sensors of the Aqua Troll 600 data sonde or the HOBO v2 data logger. Instead, they will be checked for accuracy and drift at least twice per year using an ice bath as a 0°C reference. This method was recommended to us by the HOBO

technical support team (pers. comm., 4/7/2023; details of the ice bath method are provided on the Onset/HOBO website), and by the In-Situ technical support team (per comm., 4/10/2023). Between field seasons (Dec-Feb), data sondes will be sent to In-Situ for an annual factory calibration, as recommended on p. 46 of the Aqua TROLL 600 manual. The factory calibration includes a thorough cleaning, full functionality check, and sensor adjustments to all applicable sensors over the entire calibration temperature range.

To identify potentially anomalous water quality data from the In-Situ data sonde and the HOBO data loggers, on a monthly basis raw data records (collected every 15 minutes) will be plotted against time to visually check any obvious spikes or outliers caused by possible issues such as sensor malfunction, fouling, exposure to air during extreme low tide, etc. Daily statistics (mean, minimum, maximum, and standard deviation) will also be calculated from the raw data records and plotted against time. If anomalous data spikes are identified for one parameter, other water quality parameters from the same site will be examined to determine whether a general issue may have affected multiple sensors (e.g. sonde out of water, blockage of water flow to sensors), or whether a single-sensor issue may have arisen. Where possible, water quality data will also be compared against other available data sources (e.g., nearest USGS water monitoring stations; other scientific projects occurring nearby) to identify anomalous (unexplainable) deviations. Problematic data records will be flagged in the database.

In instances where drift has been detected in a sensor (prior to monthly calibrations, or by ice bath test - see above), data will be plotted against time to visually assess whether a sudden sensor shift may have occurred (as opposed to a gradual drift). The degree of drift will be compared with data quality objective criteria specified in **Table 1-14**. Data that violate specified accuracy values defined in that table will be flagged in the database.

5.2.2 Laboratory Equipment

All lab instruments (e.g., Bio Rad CFX96 Touch Real-Time PCR Detection System, Thermo Scientific Nanodrop 2000) will undergo regular maintenance and calibration according to manufacturer recommendations.

The real-time PCR thermocycler acquired for molecular triplex assay will be maintained and calibrated according to manufacturer recommendations. The Bio Rad CFX Opus 96 Real-Time PCR system is currently in the federal acquisition process and awaiting approval. This instrument is pre-calibrated for the 3 dyes (FAM, HEX, and Cy5) required for the triplex disease diagnostic assay. The thermocycler will be maintained according to manufacturer recommendations provided in Appendix D, Maintenance and Troubleshooting of the CFX Opus Operation Manual (pg. 141) If this specific machine is not approved for purchase, a thermocycler of similar function will be acquired, maintained, and calibrated based on manufacturer's requirements by a technician.

The Thermo Scientific Nanodrop 2000 will be calibrated every 6 months according to the manufacturer specifications (Section 7-2, Diagnostics and Troubleshooting. Prior to each

sample run, a “blank” elution buffer solution will be run on the instrument to assess function and calibrate the measurements for the tested samples.

Temperatures of 4°C refrigerators as well as -20°C and -80°C freezers will be regularly monitored and logged according to Milford Laboratory standard operating protocols. If temperatures are found to be unstable or insufficient for the required protocols, samples will be transferred to backup systems until the unit can be repaired or replaced. -80°C freezers are equipped with alert systems that will be triggered when drastic temperature shifts are experienced. The -80°C freezer will undergo an annual thaw and clean out to remove frost build up to ensure efficiency and space.

Pipettes and scales will be cleaned and calibrated on an annual basis by a professional technician according to their standard procedures. If facility funds cannot support professional calibration support, scales and balances can be calibrated by Milford laboratory staff according to the respective manufacturer requirements.

Records of calibration events for both field and laboratory equipment will be stored in a dedicated calibration table of the project database. In addition, any calibration files that are automatically generated by instruments will be saved and backed up on the project server.

6. DATA MANAGEMENT

6.1 Data Assessment Procedures

Field and laboratory data will be recorded on data sheets and transcribed to digital data records on the secure NOAA server within 3 business days of the date of field collection. This will ensure all notes are correctly updated while the events are fresh in the team's memory. Digital data files will be QC'd by a second project team member alongside the data sheets to ensure all data were correctly entered within one week of data entry. Discrepancies in the data will be resolved by comparing data sheets and digital logs alongside field/lab notebooks that contain site and date specific information that could explain outliers in the data. A record of the field/lab recorder, person entering the data, and QC completion will be logged to ensure all tasks are completed prior to final data collation. Each month after QC, data will be collated with the previous data and exploratory plots updated. This data will be reviewed by the respective field and laboratory QA managers and will serve as an added check for potential error in the data collection and archiving process.

Details on data storage and archiving for public access are included in **Section 1.6.2**.

6.2 Data to be Included in QA Summary Reports

All data, up to one month prior to when the report is due, will be included in the QA reports. These data will be included in the form as archivable .csv files and the written report will include exploratory plots that show the trends in data collected. Statistical analyses will not be included in these QA summaries unless enough data points have been collected to warrant the need for statistical comparisons. **Table 6–1** summarizes the types of data that will be included in the QA Summary Reports. Final QA report at the end of the project will include all data collected with summary plots and statistical analysis.

6.3 Reporting Format

QA Summary Reports include the extent to which projects are implemented according to the stated scope of work and the methodologies specified in this QAPP in their final programmatic reports. All results meeting data quality objectives and results having satisfactory explanations for deviations from objectives will be reported. The final results will include the results of all field and laboratory quality control samples. Significant changes to the objective, scope, or methodology of environmental data collection or use of environmental technology require the review and approval of the EPA Region 1 Project Officer and the EPA Region 1 QA reviewer. Therefore, if needed, appropriate revisions to this QAPP will be completed and submitted to the EPA Region 1 Project Officer for review and approval prior to implementation of changes.

Written QA summary reports with figures will be developed in R (r-project.org) and published on a GitHub (github.com) project page for public archiving. QA data will also be archived as a .csv

format and stored on NOAA secure servers for quality assurance purposes. All reports will be submitted electronically.

Table 6–1. Data to be included in QA Summary Reports.

Data	Data Description	Reporting Method	Frequency
Geospatial Data	Monitoring site locations	GPS coordinates	During the closeout procedure and as requested by EPA.
Oyster Monitoring Data	Raw data on oyster bed density, size distribution, recruitment, reproductive state, disease prevalence and intensity (Dermo, MSX, SSO) for each site and sampling date	Raw data spreadsheets (.csv files)	During the closeout procedure and as requested by EPA.
Water Monitoring Data	Raw data on temperature, salinity, dissolved oxygen, pH, and chlorophyll a for each site and sampling date	Raw data spreadsheets (.csv files)	During the closeout procedure and as requested by EPA.

7. DATA VERIFICATION AND USABILITY

Data will be entered within 3 days of field or laboratory collection and QC'd within one week of entry by a second project team member as detailed in **Section 6.1** of this QAPP. Self assessments in the form of a monthly team QA meeting, will be completed after each field site visit to assure samples and data collection are in accordance with the methods outlined in this QAPP and detailed SOPs referenced herein. The respective QA manager for field or laboratory data (as described **Section 1.1**) will review data monthly for completeness and accuracy (determined by defined DQAOs in **Section 1.4**) so that any problems can be addressed prior to the next site visit and data collection.

7.1 Self-Assessment, Data System Audits

Self-assessment of the data will be regularly performed as described in **Sections 1.4, 6.1,** and **7**. Monthly team QA meetings will be used to regularly evaluate data quality and discuss potential solutions to improve data quality issues in the data collection, entry, assessment, and processing steps. Technical audits of each team member performing laboratory and field activities will be conducted annually by the respective QA manager by observing and documenting adherence to SOPs for data collection, sample collection, sample processing, and relevant safety precautions. Any issues in protocol implementation will be addressed with hands-on training and temporary supervision of the deficient activity until proficiency is demonstrated. Date and results of technical audits will be recorded in the project database.

8. REFERENCES

Andrews, J.D., 1988. Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effects on the oyster industry. American Fisheries Society Special Publication 18, 47–63.

Andrews, J.D., 1982. Epizootiology of late summer and fall infections of oysters by *Haplosporidium nelsoni*, and comparisons to annual life cycle of *Haplosporidium costalis*, a typical haplosporidan. J. Shellfish Res. 2, 15–32.

Aranishi, F., Okimoto, T., 2006. A simple and reliable method for DNA extraction from bivalve mantle. J. Appl. Genet. 47, 251–254. https://doi.org/DOI: 10.1007/BF03194632

Baggett, L.P., Powers, S.P., Brumbaugh, R., Coen, L.D., DeAngelis, B., Greene, J., Hancock, B., Morlock, S., 2014. Oyster habitat restoration monitoring and assessment handbook. The Nature Conservancy, Arlington, VA, USA.

Bushek, D., Ford, S.E., Allen, S.K., 1994. Evaluation of methods using Ray's fluid thioglycollate medium for diagnosis of *Perkinsus marinus* infection in the Eastern oyster, *Crassostrea virginica*. Annu. Rev. Fish Dis. 4, 201–217. https://doi.org/10.1016/0959-8030(94)90029-9

Carnegie, R.B., 2021. Chapter 2.4.5. Infection with *Perkinsus marinus*, in: Manual of Diagnostic Tests for Aquatic Animals. World Organisation for Animal Health.

Carnegie, R.B., Burreson, E.M., 2011. Declining impact of an introduced pathogen: *Haplosporidium nelsoni* in the oyster *Crassostrea virginica* in Chesapeake Bay. Mar. Ecol. Prog. Ser. 432, 1–15. https://doi.org/10.3354/meps09221

Colden, A., Latour, R., Lipcius, R., 2017. Reef height drives threshold dynamics of restored oyster reefs. Mar. Ecol. Prog. Ser. 582, 1–13. https://doi.org/10.3354/meps12362

Connecticut Bureau of Aquaculture & Laboratory Services, 2021. 2021 Statewide shellfish disease update. Bureau of Aquaculture & Laboratory Services, State of Connecticut Department of Agriculture.

Fisher, W.S., Winstead, J.T., Oliver, L.M., Edmiston, L., Bailey, G.O., 1996. Physiologic variability of Eastern oysters from Apalachicola Bay, Florida. J. Shellfish Res. 15, 543–553.

Fitzsimons, J., Branigan, S., Brumbaugh, R.D., McDonald, T., zu Ermgassen, P.S.E., 2019. Restoration guidelines for shellfish reefs. The Nature Conservancy, Carlton, Vic.

Ford, S.E., Cummings, M.J., Powell, E.N., 2006. Estimating mortality in natural assemblages of oysters. Estuaries Coast. 29, 361–374. https://doi.org/10.1007/BF02784986

Ford, S.E., Stokes, N.A., Alcox, K.A., Kraus, B.S.F., Barber, R.D., Carnegie, R.B., Burreson, E.M., 2018. Investigating the life cycle of *Haplosporidium nelsoni* (MSX): a review. J. Shellfish Res. 37, 679–693. https://doi.org/10.2983/035.037.0402

Frost, N.J., Burrows, M.T., Johnson, M.P., Hanley, M.E., Hawkins, S.J., 2005. Measuring surface complexity in ecological studies: measuring surface complexity. Limnol. Oceanogr. Methods 3, 203–210. https://doi.org/10.4319/lom.2005.3.203

Grabowski, J.H., Hughes, A.R., Kimbro, D.L., 2008. Habitat complexity influences cascading effects of multiple predators. Ecology 89, 3413–3422. https://doi.org/10.1890/07-1057.1

Harding, J.M., Powell, E.N., Mann, R., Southworth, M.J., 2013. Variations in eastern oyster (*Crassostrea virginica*) sex-ratios from three Virginia estuaries: protandry, growth and demographics. J. Mar. Biolog. Assoc. U.K. 93, 519–531. https://doi.org/10.1017/S002531541200032X

Haven, D.S., Fritz, L.W., 1985. Setting of the American oyster *Crassostrea virginica* in the James River, Virginia, USA: temporal and spatial distribution. Mar. Biol. 86, 271–282.

Hornick, K.M., Plough, L.V., 2022. Genome‐wide analysis of natural and restored eastern oyster populations reveals local adaptation and positive impacts of planting frequency and broodstock number. Evol. Appl. 15, 40–59. https://doi.org/10.1111/eva.13322

Hornick, K.M., Plough, L.V., 2019. Tracking genetic diversity in a large-scale oyster restoration program: effects of hatchery propagation and initial characterization of diversity on restored vs. wild reefs. Heredity 123, 92–105. https://doi.org/10.1038/s41437-019-0202-6

Howard, D.W., Lewis, E.J., Keller, B.J., Smith, C.S., 2004. Histological techniques for marine bivalve mollusks and crustaceans, 2nd ed. NOAA Technical Memorandum NOS NCCOS 5.

Janiak, D., 2021. MarineGEO Oyster Reef Habitat Monitoring Protocol. Tennenbaum Marine Observatories Network, MarineGEO, Smithsonian Institution.

Knoell, Alexandria, Knoell, Alee, Marcum, P., Marcum, P., Dix, N., 2021. Guana Tolomato Matanzas National Estuarine Research Reserve: Oyster spat monitoring final summary report (2015 to 2020).

Krebs, C.J., 1999. Ecological methodology, 2nd ed. Benjamin/Cummings, Menlo Park, Calif.

Lodeiros, C., Valentich-Scott, P., Chávez-Villalba, J., Mazón-Suástegui, J.M., Grijalva-Chon, J.M., 2020. Tropical and subtropical Ostreidae of the American Pacific: taxonomy, biology, ecology, and genetics. J. Shellfish Res. 39, 181. https://doi.org/10.2983/035.039.0202

Loosanoff, V.L., 1966. Time and intensity of setting of the oyster, *Crassostrea virginica*, in Long Island Sound. Biol. Bull. 130, 211–227. https://doi.org/10.2307/1539698

Mackin, J., 1962. Oyster disease caused by *Dermocystidium marinum* and other microorganisms in Louisiana. Publ Inst Mar Sci Univ Tex 7, 132–229.

Mann, R., Southworth, M., Carnegie, R.B., Crockett, R.K., 2014. Temporal variation in fecundity and spawning in the Eastern oyster, *Crassostrea virginica*, in the Piankatank River, Virginia. J. Shellfish Res. 33, 167–176. https://doi.org/10.2983/035.033.0116

McFarland, K., Hare, M.P., 2018. Restoring oysters to urban estuaries: Redefining habitat quality for eastern oyster performance near New York City. PLoS ONE 13, e0207368. https://doi.org/10.1371/journal.pone.0207368

McFarland, K., Rumbold, D., Loh, A.N., Haynes, L., Tolley, S.G., Gorman, P., Welch, B., Goodman, P., Barnes, T.K., Doering, P.H., Soudant, P., Volety, A.K., 2022. Effects of freshwater release on oyster reef density, reproduction, and disease in a highly modified estuary. Environ Monit Assess 194, 96. https://doi.org/10.1007/s10661-021-09489-x

National Research Council, 1980. The International Mussel Watch: Report of a Workshop. Washington, D.C: The National Academies Press.

Piesz, J.L., Scro, A.K., Corbett, R., Markey Lundgren, K., Smolowitz, R., Gomez-Chiarri, M., 2022. Development of multiplex qPCR for quantification of three protozoan parasites of the eastern oyster *Crassostrea virginica*. Dis. Aquat. Org. 151, 11–121. https://doi.org/10.3354/dao03694

Ray, S.M., 1954a. Biological studies of *Dermocystidium marinum*, a fungus parasite of oysters. Rice University, The Rice Institute Pamphlet Special Issue.

Ray, S.M., 1954b. Studies of the occurrence of *Dermocystidium marinum* in young oysters., in: Proc. Natl. Shellfish. Assn. pp. 80–92.

Reidenbach, M.A., Limm, M., Hondzo, M., Stacey, M.T., 2010. Effects of bed roughness on boundary layer mixing and mass flux across the sediment-water interface: turbulence and mass flux at the sediment. Water Resour. Res. 46. https://doi.org/10.1029/2009WR008248

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

Sunila, I., Kenyon, H., Rivara, K., Blacker, K., Getchis, T., 2016. Restocking natural beds with remote-set disease-resistant oysters in Connecticut: a field trial. J. Shellfish Res. 35, 115–125. https://doi.org/10.2983/035.035.0113

Tarnowski, M., 2021. Maryland Oyster Population Status Report: 2021 Fall Survey. Maryland Department of Natural Resources, Annapolis MD.

Volety, A., Perkins, F.O., Mann, R., Hershberg, P., 2000. Progression of diseases caused by the oyster parasites, *Perkinsus marinus* and *Haplosporidium nelsoni*, in *Crassostrea virginica* on constructed intertidal reefs. J. Shellfish Res.

Whitlock, M.C., Schluter, D., 2019. The analysis of biological data, Third edition. ed. Macmillan Learning, New York.

9. APPENDICES

Appendix A – Oyster Tissue Processing & Preservation SOP

1 Purpose

The purpose of this document is to provide a standard method of processing live oysters and preserving tissue samples for various diagnostic assays using sterile techniques. This protocol enables multiple diagnostics on a single oyster reducing the number of sacrificed animals.

2 Scope

This SOP applies to Northeast Fisheries Science Center, Milford Laboratory staff processing live eastern oysters collected for the Long Island Sound Study (LISS) funded oyster disease project and being preserved for microbial culture, histology, and molecular diagnostics.

3 Definitions/Acronyms

RFTM - Ray's Fluid Thioglycollate Medium

4 Reagents/Media

- 95% Ethanol
- 70% Ethanol
- Penicillin-Streptomycin Solution
    - Penicillin G Sodium Salt 98% (1636.6 IU/mg), 455690050, Acros Organics
    - Streptomycin sulfate (720 IU/mg), 61224-0500, Acros Organics
- Nystatin Solution
    - Nystatin 5g Amber Glass, BP29495, Fisher BioReagents
    - Deionized water
- Bleach
- Davidson's Fixative, s2250-1GL, Poly Scientific R & D Corp, Bay Shore, NY
- RFTM
    - Sodium chloride (NaCl) [7647-14-5] S-3014. FSigma Chemical Company, St. Louis, MO
    - Fluid Thioglycollate Medium (FTM), Powder, R45352, Remel

5 Supplies/Materials

- Gloves (Nitrile and Shucking)
- Lab coats
- Lab markers/pens/pencils
- Scale
- Bristle Brush (for cleaning oysters)
- Paper towels
- Spray bottles for bleaching equipment
- Freshwater
- Caliper
- Sand
- Tin foil or weigh boats
- Trays

- Shucking knife
- Dissection tools - scalpel, scissors, probes, and forceps
- Pathology blades
- Glass jars
- Bunsen burner or ethanol lamp flame
- Delicate task laboratory wipes
- Histology cassettes
- 1.5 mL Microcentrifuge tubes PCR clean
- 15mL glass culture tubes and screw caps
- Pipette set (1000µL, 200µL, 50µL, 10µL, 2µL)
- Pipette tips (1000µL, 200µL, 50µL, 10µL, 2µL)

6 Equipment

- Refrigerator

7 Safety Precautions

Laboratory members will wear gloves to process the samples. Nitrile gloves will be worn when handling tissue and shucking gloves will be worn when shucking/opening live oysters. Members will follow standard laboratory procedures in the event of an accident (i.e. spilling chemicals, eye contamination, chemical burns) as well as for disposal of chemicals and any hazardous wastes. Chemicals will be clearly labeled and kept under a biological safety cabinet if necessary. Steps within the process will also be conducted under a fume hood (see below). Blades and scalpels will be kept covered when not in use and disposed of in a sharps container. Bunsen burners/Ethanol lamps will not be left unattended and must be turned off. Lab members will have knowledge of the location of the nearest first-aid kit. All Safety Data Sheets (SDS) for chemicals can be found in the binders on lab doors.

8 Laboratory Quality Control

Laboratory members will wear gloves (nitrile or shucking) and lab coats to reduce the risk of contamination. All equipment will be sterilized before and after use, as well as in between individual organisms. Tubes and histology cassettes will be pre-labeled prior to processing tissue to streamline the process and prevent samples from being incorrectly identified. There will be designated stations/lab areas for each process. Multiple members of the lab will process samples at the same time to ensure everyone is using the same methods and corrections can be made when necessary. Guidelines for measuring and other steps in the process will be discussed between members prior to start to ensure uniformity. For tissue sectioning for histology, there will be a gauge at the station to ensure proper thickness of the cross section and photos will also be posted in the area for easy reference.

9 Preparation

1. At least one day prior to field sampling, examine the lab to make sure the area is ready for processing. There should be adequate space in the sharps containers and chemical waste bins. If there is not, properly dispose of materials to create a clear working environment. Biological trash bins should also be emptied, autoclaved, and properly disposed of. Bins should not exceed 75% full.
2. Check the supply list for the procedures and take inventory. Make sure there are adequate amounts of supplies and reagents needed.

3. All laboratory stations should be set up and cleared of unnecessary equipment for easy work flow.
4. Calibrate any equipment used in the procedures (i.e. calipers, scales, etc)
5. Cut and label foils for numbering oysters and keeping track.
6. Label all microcentrifuge tubes for qPCR, jars and cassettes for histology, and culture tubes for RFTM.
    a. Cassettes for histology should be labeled using a pencil.
    b. Microcentrifuge tubes should be labeled by printing full identifying details on waterproof paper and taping to the tube with packing tape (this will assure that split ethanol does not remove labels written with marker and that all information is legible).
7. Prepare any media/reagents needed for processing. Pre-fill labeled microcentrifuge tubes with 95% ethanol for the qPCR samples. Prepare RFTM, penicillin-streptomycin solution, and nystatin solution (**Appendix B**) and pre-fill labeled culture tubes. Organize and all pre-label all cassettes and prepare Davidson's fixative (**Appendix E**) in jars to be stored.
8. All data collection sheets should be organized, dated, and printed or electronically available for data recording.

10 Procedures

1. Upon arrival at the laboratory, animals are kept refrigerated until ready for processing. Processing should occur within 48hrs of collection to maintain integrity and quality of tissue.

2. Using cold freshwater, scrub animals with the bristle brush and rinse animals well. Remove all fouling organisms with a shucking knife if necessary.

3. Scrub hinges with a toothbrush and 6% bleach to sanitize the area. Use a paper towel to remove excess water and set aside on a clean paper towel and individually labeled piece of foil.

4. **Shell Measurements**:

    a. **Shell height** is measured from the hinge to the free edge (anterior- posterior axis) of the shell to the nearest tenth of a millimeter (Figure 1).

    b. **Shell length** is measured along the widest point of the dorsal- ventral axis.

    c. **Shell width** is measured from the right to left shell.

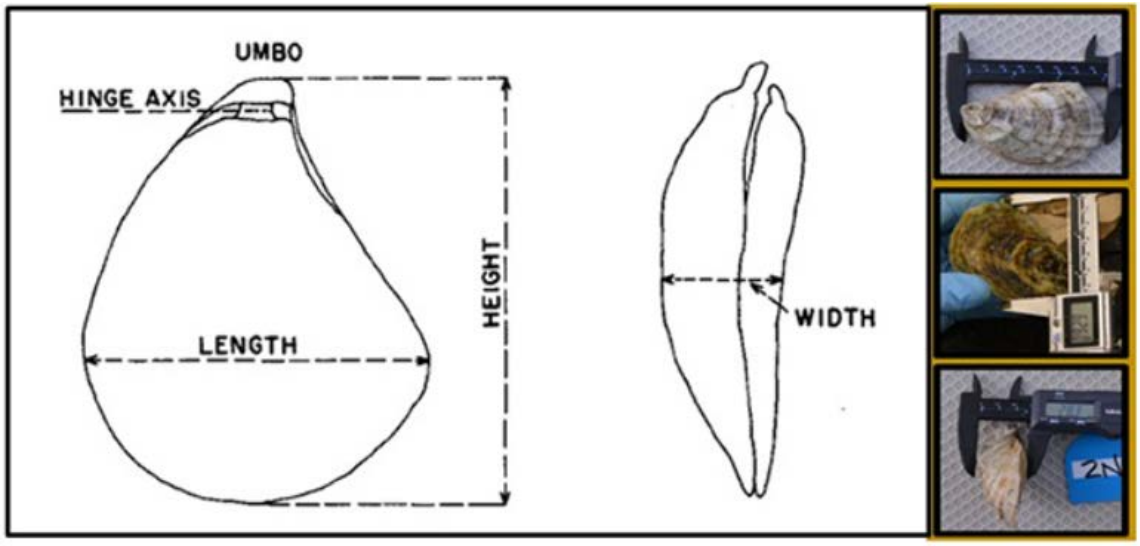


Figure 1. Demonstration of shell measurements on external anatomy of an oyster shell.

5. Measure the **whole oyster wet weight**. First, tare the scale with the paper towel and foil piece in place. Place the body of the oyster onto the scale and measure the wet weight in grams to the nearest tenth (0.1 g).

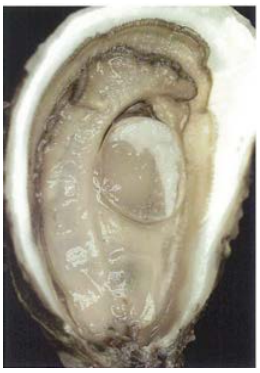
6. Using a sterile shucking knife, open each oyster by severing the adductor muscle at its attachment to the upper (right) shell. *Be sure to keep the body intact (not damaging any tissue with the shucking knife) and in the cup of the left shell* (Figure 2).

Figure 2. Shucked Oyster

   a. All tools are sterilized between individuals following the steps listed below:
      i. Freshwater dip
      ii. Bleach and sand dip to remove tissue
      iii. Freshwater dip
      iv. 95% ethanol dip
      v. Ethanol is burned off using a flame (allow to cool before reusing)

7. After shucking the animal, visually inspect the body and assign a **body condition rating** (Table 1). This will serve as an indicator of the oyster's overall health status

Table 1. Oyster condition rating key (from Histological Techniques for Marine Bivalves Mollusks and Crustaceans. NOAA Technical Memorandum NOS NCCOS 5, 2004.)

Rating	Description of visual observations	Example image
Fat	Body usually fills the shell and the tissue is plump and firm. Often the gonads are ripe and creamy-tan in color.	
Medium	Generally smaller with more fluid present in the shell. The tissue is softer and more flat. Overall color is tan.	
Watery	Body is small and limp, surrounded by significant fluid. Color can be grayish and appear translucent.	

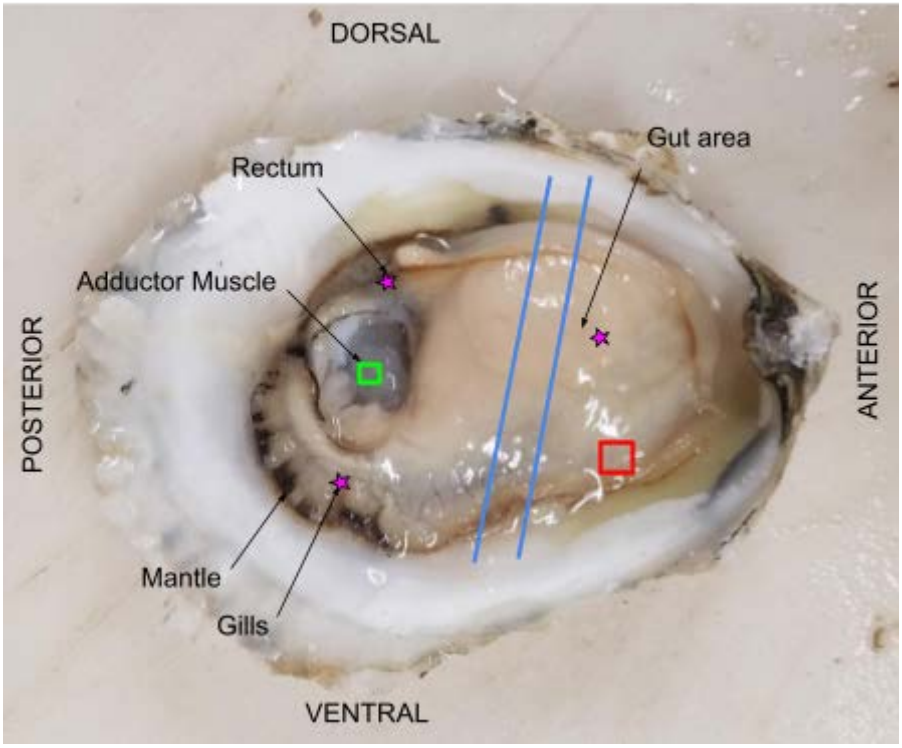


Figure 3: Anatomy of *Crassostrea virginica* for aid in dissection. Blue parallel lines are the location at which histological sectioning should occur. The Red square is the location at which tissue should be extracted for RFTM preservation. Pink stars are the locations at which tissue should be taken for qPCR samples. Green square is the location at which adductor muscle tissue should be taken for archiving.

*** All individuals will be preserved for each of the following processes ****

8. For DNA Extractions for triplex qPCR diagnostic assay and tissue archiving:

    a. Using sterile instruments, gill, gut and rectal tissues are collected and stored together in 1.5mL microcentrifuge tubes in 95% ethanol. To enable additional samples to be collected from the same individual, tissues must be precisely excised from specific locations noted in Figure 3 and only 0.5mg of tissue should be removed (Figure 4). ***A duplicate sample from each oyster will be collected and stored for backup ****

    b. Using the same instruments, a small 5 x 5mm section of adductor muscle tissue is collected and stored in a 1.5mL microcentrifuge tube in 95% ethanol.

    c. Tissues in ethanol are stored in the ethanol safe freezer in room 17 for short term storage and in the -80°C in the Building 1 garage for long term storage until DNA extraction (**Appendix C**) and qPCR analysis.

d. Instruments used to retrieve tissues are sterilized in between oyster samples by the same methods described in section 6a.

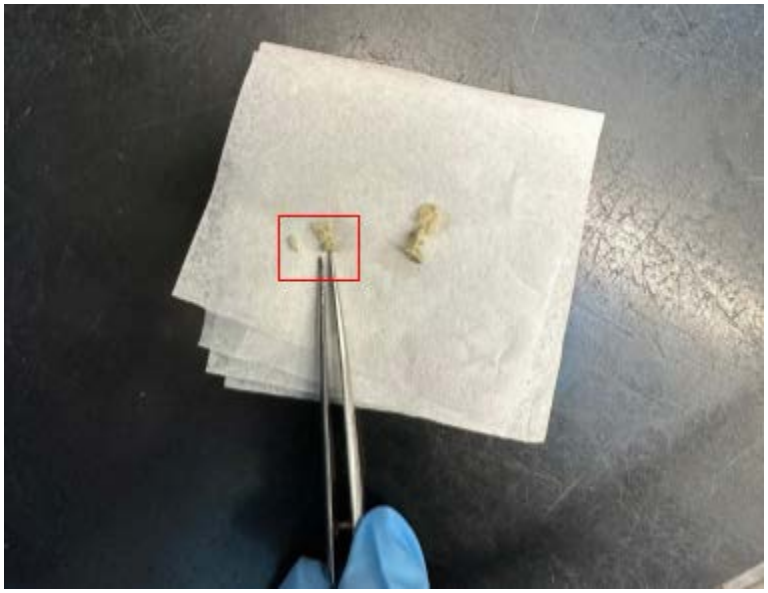


Figure 4: Size comparison of 0.5mg of gut, mantle, and gill tissue samples from eastern oysters for DNA extraction. Size of each tissue type is no larger than the tip of a fine-point forceps.

9. For RTFM:

    a. Using sterile dissecting scissors and forceps, a 5x5mm piece of mantle-edge tissue is excised from just over the palps. See Figure 3.

    b. Place the tissue in a culture tube containing 5 mL RFTM to which 0.5 mL of the Penicillin-Streptomycin solution and 50 µL Nystatin solution has been added (**Appendix B**). Use a sterile probe to fully submerge the tissue in the bottom third of the RFTM.

    c. Screw cap back on tightly.

    d. Culture tubes with tissues are placed in the dark at room temperature and incubated for at least 5 days.

    e. If not analyzed within 7 days of processing, the tube should be placed in a refrigerator in the dark (can be kept up to 3 months without degradation).

    f. For tissue analysis, see **Appendix B**.

10. For Histology:

    a. Following sample collection for qPCR and RFTM analyses, the remaining oyster shell and body will be transferred to a fume hood to be processed for histology.

    b. Use a shucking knife to carefully remove the oyster body from the shell and place on a flat surface. The shell can be discarded in the regular waste stream according to Milford Laboratory protocol.

Figure 5. Demonstration of the first diagonal cut across the body of the oyster for histology.

Figure 6. Demonstration of second cut to create a cross-section of oyster tissue for histology.

Figure 7. Cross section placed into histology cassette.

c. Using a razor blade, make a single clean diagonal cut across the body of the animal (Figure 3, Figure 5).

d. Make a second diagonal cut on the bottom half (without the adductor muscle) of the oyster (Figure 3, Figure 6). *The cross-section should be 4mm thick and include portions of the mantle, stomach, digestive tubules, gonads, kidney, and gills*.

e. Take the cross section and place it into the cassette (Figure 7), which was prelabeled using pencil.

f. Place the cassettes with tissue into Davidson's Fixative (**Appendix E**) and store in the refrigerator for a minimum of 24 hours (maximum 48 hours).

    i. After the fixation period is complete, drain the fluid and fill with 70% ethanol to cover all of the cassettes. Add the date of ethanol transfer to the bottle label and initial. *This is a wash step and tissue should be soaked for at least 24hrs.*

    ii. Cassettes should be soaked in ethanol for at least 24 hours before being embedded in paraffin, but they can be stored in a cool secure location for several weeks while preserved in ethanol.

g. For processing and slide preparation, see **Appendix E**.

11. All samples should be dated and documented in the records database.

11 Data Quality Control

Laboratory data will be recorded on data sheets and transcribed to digital data records on the secure NOAA server within 3 business days of the date of collection and processing. This will ensure all notes are correctly updated while the events are fresh in the team's memory. Digital data files will be QC'd by a second individual alongside the data sheets to ensure all data was correctly entered within one week of data entry. Discrepancies in the data will be resolved by comparing data sheets and digital logs alongside lab notebooks that contain site and date specific information that could explain outliers in the data. A record of the lab recorder, person entering the data, and QC completion will be logged to ensure all tasks are completed prior to final data collation. Each month after QC, data will be collated with the previous data and exploratory plots updated. This will serve as an added check for potential error in the data collection and archiving process.

All laboratory data sheets will be scanned and filed on the NOAA server in the project folder. Physical copies will also be filed for archiving at the NOAA Milford Laboratory. Purely digital files (eg. sonde data, photos, etc.) will be uploaded directly to the NOAA server which is backed up regularly.

END OF SOP

Appendix B – Ray's Fluid Thioglycollate Medium (RFTM) assay for detection of *Perkinsus marinus* SOP

1 Purpose

The purpose of this SOP is to provide concise guidance and standard methodology on how to determine presence and infection intensity of *Perkinsus marinus* in oyster tissue using the Ray's Fluid Thioglycollate Medium (RFTM) assay.

2 Scope

This SOP applies to Northeast Fisheries Science Center, Milford Laboratory staff performing the RFTM assay on tissues from eastern oysters, *Crassostrea virginica* collected for theLong Island Sound Study (LISS) funded oyster disease project.

3 Definitions/Acronyms

RFTM - Ray's Fluid Thioglycollate Medium

4 Reagents/Media

- Fluid Thioglycollate Medium (FTM), Powder, R45352, Remel
- Lugol's Iodine (5g Iodine per 1000mL DI H20), R40029, Remel
- Penicillin G Sodium Salt 98% (1636.6 IU/mg), 455690050, Acros Organics
- Sodium chloride (NaCl) [7647-14-5] S-3014. Sigma Chemical Company, St. Louis, MO
- Streptomycin sulfate (720 IU/mg), 61224-0500, Acros Organics
- Nystatin 5g Amber Glass, BP29495, Fisher BioReagents

5 Supplies/Materials

- Flasks (1L and 250mL)
- Distilled water
- Deionized water (DI)
- Stirring rods
- Autoclave Mitts
- 50mL falcon tubes
- Automatic Repipet, Barnstead/Thermolyne Repipet II (9010), 13-687-62B. Fisher Scientific, Pittsburgh, PA.
- Coverslips, various sizes, Corning Inc. Corning , NY.
- Culture Tubes with Screw Cap, Pyrex, 14-932A. Fisher Scientific, Pittsburgh, PA.
- Dissecting Tools- scissors, scalpels, probes, forceps
- Razor blades
- Microscope Slides, Frosted, 2948-78X25. Corning Inc., Corning, NY.
- Pasteur Pipettes, 13-678-20A. Fisher Scientific, Pittsburgh, PA.
- Repipet, 1 ml capacity, adjustable volume, P-1000. Rainin Instrument Co., Woburn, MA.
- Repipet tips, Redi-Tip Universal, 21-197-8F. Fisher Scientific, Pittsburgh, PA.
- Vortex mixer, Vortex-Genie 2 Model G-560, 12-812. Fisher Scientific, Pittsburgh, PA.

6 Equipment

- Heat plate
- Autoclave
- Refrigerator
- Scale
- Compound Microscope
- Alcohol lamp

7 Safety Precautions

Laboratory members will wear gloves and protective eyewear when using chemicals. Members will follow standard laboratory procedures in the event of an accident (i.e. spilling chemicals, eye contamination, chemical burns) as well as for disposal of chemicals and any hazardous wastes. Chemicals will be clearly labeled and kept under a fume hood if necessary. Blades and scalpels will be kept covered when not in use and disposed of in a sharps container. Bunsen burners/EtOH lamps will not be left unattended and must be turned off when not in use. Lab members will have knowledge of the location of the nearest first-aid kit. All Safety Data Sheets (SDS) for chemicals can be found in the binders on lab doors.

8 Laboratory Quality Control

Laboratory members will wear gloves and lab coats to reduce the risk of contamination. All equipment will be sterilized before and after use, as well as in between individual organisms. Tubes and slides will be pre-labeled prior to microscopy to streamline the process and prevent samples from being incorrectly identified. There will be designated stations/lab areas for each process. Multiple members of the lab will be processing samples at the same time to ensure everyone is using the same methods and corrections can be made when necessary. Additionally, multiple lab members will read slides to ensure agreement and consistency in results. Guidelines for assigning infection intensity and other steps in the process will be discussed between members prior to start to ensure uniformity. Samples will also be done in subsets at regular intervals to increase accuracy.

9 Preparation

1. At least one day prior to performing RFTM assay, examine the lab to make sure the area is ready for processing. There should be adequate space in the sharps containers and chemical waste bins. If there is not, properly dispose of materials to create a clear working environment. Biological trash bins should also be emptied, autoclaved, and properly disposed of. Bins should not exceed 75% full.

2. Take inventory of required supplies. Make sure there are adequate amounts of supplies and reagents needed.

3. Ensure any glassware or dissection tools have been properly cleaned and sterilized for the procedures.

4. All laboratory stations should be set up and cleared of unnecessary equipment for easy work flow.

5. If needed, calibrate any equipment used in the procedures (i.e. scales, etc).
6. Make sure all equipment is running properly such as heat plates and microscopes.
7. Make sure all culture tubes have been pre-labeled (oyster sample ID, Site, Date).
8. **RFTM (steps 1-3 in procedures) and preparation of culture tubes should be done at least one day prior to sampling.**
9. All data collection sheets should be organized, dated, and printed or electronically available for data recording.
10. All data collection sheets should be organized, dated, printed or electronically available for data recording.

10 Procedures

Steps 1-3 are to be done at least 1 day prior to sampling and tissue processing.

1. Ray's (1952) Thioglycollate Medium (RFTM) preparation
    a. In a flask, add 22 g of NaCl, 29.3 g of dehydrated Fluid Thioglycollate medium (FTM) into 1 L of distilled water.
    b. Put the flask on a heat plate at the highest setting and stir continuously with the stir bar until dissolved.
    c. The mixture will be pink and change to a golden-yellow color as it begins to boil.

Figure 1. Image showing the media color just prior to boiling. Votex shows an indicator of the speed of the stir bar.

    d. When it begins to boil, immediately take the mixture off of the heat plate. *Do not let it overflow from boiling*.
    e. Place the flask into a tub of room temperature water to cool down.
    f. After the flask is cooled to the touch, dispense 5mL of solution into each 15mL culture tube. Screw the caps on loosely.

g. The tubes with medium are then autoclaved for 15 minutes at 121℃ (15psi) on the slow exhaust setting.

h. When autoclaving is finished, allow tubes to fully cool to room temperature, then screw caps to seal. Place the culture tubes in the refrigerator and in the dark until ready for use.

   i. Unused autoclaved tubes of RFTM can be stored for many months in the dark without deterioration.

   ii. Discard them if they become cloudy or the RFTM congeals.

i. *IMPORTANT: RFTM maintains anaerobic conditions in the culture tube as well as providing nutrients and an appropriate osmotic environment. Therefore, tubes are kept sealed tightly and only opened briefly to add antibiotics and tissue as described below. After tissue is added, seal the tubes and return them immediately to the dark for the tissue to incubate*.

   i. There is a color change indicator that is yellow when anaerobic and will change to pink when conditions become aerobic (due to the presence of resazurin). If the tubes demonstrate >30% oxidation (color change to pink), they can be reheated 1 time. Heat tubes with caps loosened for approximately 10 minutes in a boiling water bath (100℃) until the pink disappears. Cool to <45℃ before inoculating with tissue.

2. Antibiotic Solution Preparation

   a. Add 0.33 g of Streptomycin Sulfate and 0.159 g of Penicillin G to 500 ml of sterile by autoclaving, deionized (DI) water in a 1L flask

   b. Swirl lightly until powder is dissolved.

   c. Keep the solution refrigerated until use and can be stored for several months.

   d. The solution can maintain potency at 4℃ (refrigeration) for 1-2 weeks. For larger batches aliquot desired amount (15mL) and store the remaining in the freezer (-20°C) until needed. The solution will maintain potency in the freezer for ~6 months.

3. Nystatin Solution Preparation

   a. In a small falcon tube mix 10mg of nystatin powder with 10mL of sterile DI water. (.1g/100mL).

   b. Mix ingredients well.The solution can be stored at 4℃ (refrigeration) for up to 2 weeks.

4. Lugol's Iodine Solution for staining

   a. Add 4g of potassium iodide and 2g of iodine crystals into 100 ml of distilled water and dissolve.

   b. Allow to stand for 24hrs and filter.

c. Store solution in a dark bottle at room temperature to avoid particle precipitation.

   i. The solution remains stable for many weeks but should be filtered occasionally to remove particles that may precipitate. These particles may be confused with *P. marinus* hypnospores by less experienced slide readers although they are disks, not spheres; they are always black, never blue; and they are birefringent.

5. Inoculation and Incubation of oyster tissues in culture tubes

   a. Add 0.5mL of the Penicillin-Streptomycin and 50µL Nystatin to each prepared and prelabeled culture tube containing 5 mL RFTM

   b. Using sterile dissecting scissors and forceps, a 5x5mm piece of mantle-edge tissue is excised from just over the palps. (Figure 1)

   c. Place the tissue in a culture tube containing 5 mL RFTM to which 0.5mL of the Penicillin-Streptomycin and 50µL nystatin has been added. Use a sterile probe to fully submerge the tissue in the bottom third of the RFTM.

   d. Screw cap back on tightly.

   e. Incubate culture tubes with tissues in the dark at room temperature and for at least 5 days.

   f. If not analyzed within 7 days of processing, the tube should be placed in a refrigerator in the dark (can be kept up to 3 months without degradation).

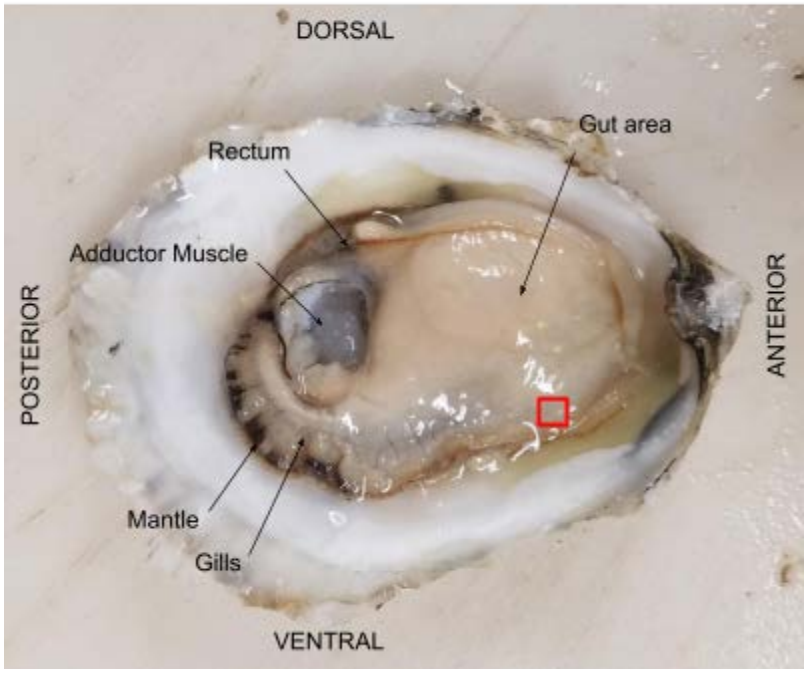


Figure 1. Anatomy of *Crassostrea virginica* for aid in dissection. The Red square is the location at which tissue should be extracted for RFTM preservation.

6. Tissue Analysis/Diagnostics

    a. After the tissue incubation period, remove the oyster tissue from the RFTM using a sterile probe and place it on a microscope slide.

    b. Tease apart the tissue sample using a sterile probe to ensure even staining with Lugol's iodine solution. ** Instruments are ethanol and flame sterilized between samples. See **Appendix A**. **

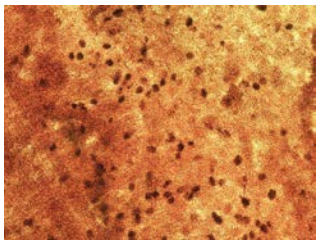
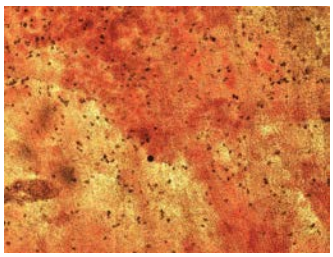
    c. Add 1-2 drops of Lugol's Iodine solution to the tissue with a Pasteur pipette and then cover with a cover slip.

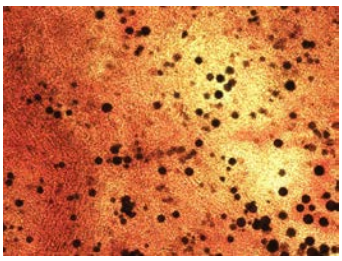
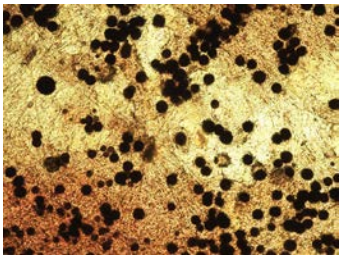
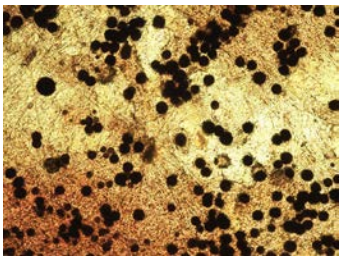
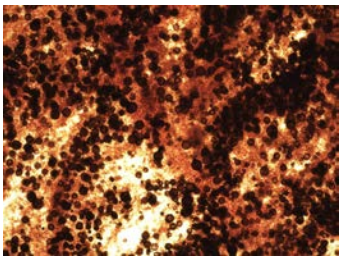
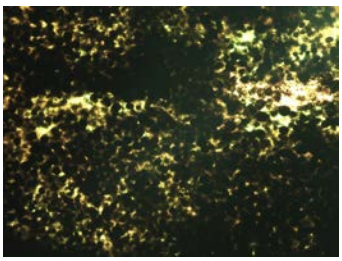
    d. Examine microscopically on a compound microscope. Entire tissue is examined under the microscope at 10x, 40x, and 100x to visualize *P. marinus* hypnospores. RFTM will enlarge parasite hypnospores of all sizes and Lugol's Iodine will stain hypnospores black. The size range for hypnospores is 5-300µm. Hypnospores are spherical in shape and to be carefully differentiated between parasite and debris.

    e. Presence is recorded and infection intensity is assigned according to the Mackin Scale (Table 1)

    f. Microscope slides and coverslips are discarded in the sharps container after use.

Table 1. Mackin Scale (Ray 1954a, 1954b). Ray's Fluid Thioglycollate Method images from Ray, S.M.

Infection intensity	Numerical Value	Description	
Negative (N)	0	No hypnospores	
Very light (VL)	0.5	1-10 hypnospores	 (note single dermo cell among many brown cells)

Light (L)	1	11-125 hypnospores; less than 25% of the tissue is hypnospores	
Light/Moderate (LM)	2	25% of tissue is hypnospores	
Moderate (M)	3	50% of tissue is hypnospores	
Moderately heavy (MH)	4	75% of tissue is hypnospores	
Heavy (H)	5	>75% of tissue is hypnospores to 100%	

g. Calculations of prevalence and intensity will be reported using the equations below:

$$Prevalence\ \% = \frac{\#\ of\ infected\ individuals\ per\ site}{total\ \#\ of\ individuals\ collected\ per\ site}$$

$$Weighted\ Prevalence\ \% = \frac{Sum\ of\ intensity\ scores}{total\ \#\ of\ individuals\ collected\ per\ site}$$

$$Intensity\ (population) = \frac{Sum\ of\ intensity\ scores}{\#\ of\ individuals\ infected\ per\ site}$$

h. See **Appendix D** for molecular approach to quantification of *P. marinus* DNA in oyster tissue.

7. Results reporting

   a. Results will be recorded using a data sheet inputted into the project database.

8. Clean up/ Disposal of Tissue

   a. Used slides with tissue and coverslips will be disposed of in the sharps container.

   b. The spent tubes with inoculated medium are then autoclaved with the caps loosely fastened for 15 minutes at 121℃ (15psi) on the slow exhaust setting. Once the liquid is cool, it can be flushed down a sink that discharges to the sewage treatment line.

11 Data Quality Control

Laboratory data will be recorded on data sheets and transcribed to digital data records on the secure NOAA server within 3 business days of the date of collection and processing. This will ensure all notes are correctly updated while the events are fresh in the team's memory. Digital data files will be QC'd by a second individual alongside the data sheets to ensure all data was correctly entered within one week of data entry. Discrepancies in the data will be resolved by comparing data sheets and digital logs alongside lab notebooks that contain site and date specific information that could explain outliers in the data. A record of the lab recorder, person entering the data, and QC completion will be logged to ensure all tasks are completed prior to final data collation. Each month after QC, data will be collated with the previous data and exploratory plots updated. This will serve as an added check for potential error in the data collection and archiving process.

All laboratory data sheets will be scanned and filed on the NOAA server in the project folder. Physical copies will also be filed for archiving at the NOAA Milford Laboratory. Purely digital files (eg. sonde data, photos, etc.) will be uploaded directly to the NOAA server which is backed up regularly.

12 References

Ray, S. M. 1954. Biological studies of *Dermocystidium marinum*, a fungus parasite of oysters. Rice University.

Ashton-Alcox, K.A., Kim, Y., and Powell, E.N. *Perkinsus marinus* Assay protocol. Haskin Shellfish Research Laboratory.

13 Appendix

A. Standard methods for sterilization of dissection tools

1. All tools are sterilized between individuals following the steps listed below:
    i. Freshwater dip
    ii. Bleach and sand dip to remove tissue
    iii. Freshwater dip
    iv. 95% ethanol dip
    v. Ethanol is burned off using a flame (allow to cool before reusing)

END OF SOP

Appendix C – DNA Extractions SOP

1 Purpose

The purpose of this document is to provide concise guidance on performing DNA extractions on oyster gill, gut, and rectal tissue for downstream molecular analysis.

2 Scope

This SOP is pertaining to the Northeast Fisheries Science Center, Milford Laboratory staff processing eastern oyster tissue (*Crassostrea virginica*) for molecular detection (qPCR) of diseases in Long Island Sound funded by the Long Island Sound Study (LISS).

3 Reagents/Media

- Urea buffer
    - Urea, powder, U5378-500G, Sigma Aldrich (Stored at room temperature)
    - Tween 20, P1379-10mL, 9005-64-5, Sigma Aldrich (Stored at room temperature)
    - Triton-X 100, 1001412355, Sigma Aldrich (Stored at room temperature)
    - Chelex 100, BT Chelex 100 Resin, 143-2832, BioRad (Stored at room temperature)
- 100TE buffer
    - Tris Base, White Crystals or Crystalline powder, BP152-500, Fisher Scientific (Stored at room temperature)
    - EDTA, ED285-1KG, Sigma Aldrich (Stored at room temperature)
    - Sterile water
- 10T0.1E buffer
    - EDTA
    - Tris-HCl
    - Sterile water
- Ammonium acetate for molecular biology, ≥98%, A1542-500G, Sigma Aldrich (Stored at room temperature)
- Proteinase K
- Ethanol (EtOH) (Stored in the safety shed behind building 1 - flammable)
- bleach
- Elution buffer

4 Supplies/Materials

- Nitrile gloves
- Lab coat
- Pipette set
- Pipette tips (1000µl, 200µl, 20µl, 2µl)
- Scale (2,200 g)
- Weigh boats (3in x 3in, 6in x 6in)
- Scoop
- 250ml beaker
- PCR water: UltraPure DNase/RNase-Free Distilled Water (PCR water), 10977023, Invitrogen, USA
- Strip tubes: USA Scientific TempAssure 0.2ml PCR 8-tube strips Cat. No. 1402-4700

- Stir bar
- 1.5mL microcentrifuge tubes
- thermometer
- delicate task wipe
- paper towels
- dissection tools- probes, forceps, scissors, scalpels

5 Equipment

- Autoclave
- Heat block
- Vortex
- Centrifuge
- Nanodrop (need specifics)
- -20℃ freezer
- -80℃ freezer

6 Safety Precautions

Laboratory members will wear gloves and protective eyewear when using chemicals. Members will follow standard laboratory procedures in the event of an accident (i.e. spilling chemicals, eye contamination, chemical burns) as well as for disposal of chemicals and any hazardous wastes. Chemicals will be clearly labeled and kept under a chemical fume hood if necessary. Blades and scalpels will be kept covered when not in use and disposed of in a sharps container. Bunsen burners/EtOH lamps will not be left unattended and must be turned off. Lab members will have knowledge of the location of the nearest first-aid kit. Safety Data Sheets (SDS) are located in the binder on the door of each lab and digital copies can be found in the shared lab Google Drive folder.

7 Laboratory Quality Control

Laboratory members will wear gloves and lab coats to reduce the risk of contamination. All equipment will be sterilized before and after use, as well as in between individual organisms. There will be designated stations/lab areas for DNA extractions. All new personnel will train alongside an experienced team member before independently executing the required tasks. A printed copy of this SOP will be available where the work is to be performed, and a digital copy will be available to all project personnel. To ensure sterile techniques, every 25-30 samples will include a blank or negative control that does not contain tissue. This control will go through the extraction process and will help identify if any contamination is occuring.

8 Preparation

1. At least one day prior to starting DNA extractions, examine the lab to make sure the area is ready for processing. There should be adequate space in the sharps containers and chemical waste bins. If there is not, properly dispose of materials to create a clear working environment. Biological trash bins should also be emptied, autoclaved, and properly disposed of. Bins should not exceed 75% full.
2. Check the supply list for the procedures and take inventory. Make sure there are adequate amounts of supplies and reagents needed. If reagents are in insufficient quantities, prepare them according to the procedures outlined in section 9.1.

3. All laboratory stations should be set up and cleared of unnecessary equipment for easy work flow.
4. Pre-label first set of 1.5mL microcentrifuge tubes for the process (section 2a) with the following information
    a. Number the tubes 1 through X based on the total number of samples being extracted
    b. Date of extraction
    c. Keep a log of which number corresponds to which sample ID in the lab notebook
    d. Label one tube as “Blank”, and include the date.
5. Pre-label final set of 1.5mL microcentrifuge tubes for the process with the following information:
    a. Unique specimen ID (numerical)
    b. Date of Collection (MM/DD/YYYY)
    c. Site
    d. Media specifics: Extracted DNA
6. Pre-label a set of PCR strip tubes for the DNA concentrated to a standard 100ng/3µL per sample. Individual tubes will only be labeled with the unique specimen ID and “DNA”.
7. Obtain the amount of ethanol needed for section 2 of procedures on Day 2 and place it in the freezer.
8. Locate prepared oyster tissue preserved in 95% Ethanol and make a list of the sample subset that will be extracted to keep track of processed samples.
9. Immediately prior to performing work, the workstation should be sprayed with 70% ethanol and wiped down to clean away any potential contaminants.

9 Procedures

1. Reagent solutions (Should be prepared in advance and can be made in batches with long term storage)

a. <u>4M Urea buffer</u> (makes 100mL)
    i. Add the following ingredients to a 250mL beaker.
        1. 100mL sterile, PCR water
        2. 24g Urea
        3. 1mL Tween 20
        4. 1mL Triton-X 100 (Note: very viscous, slowly pipette)
        5. 5g Chelex 100
    ii. Using a stir bar, mix for a few minutes.
    iii. Leave stir bar in the beaker, cover beaker with aluminum foil, and autoclave solution for 20 minutes at 121℃ (15psi) on the slow exhaust setting
    iv. Allow the solution to completely cool before adding 5mg of Proteinase K.

v. Aliquot 20mL solution into 50mL sterile tubes. Keep tubes with urea buffer refrigerated until use.

b. 100TE buffer (makes 50mL)
   i. Add the following ingredients to a 50mL sterile falcon tube.
      1. 6.057g 1M tris
      2. 10mL 100mM EDTA (of 0.5M stock)
   ii. 40mL sterile, PCR water
   iii. Cap tube and gently invert to mix well.
   iv. Store at room temperature.

c. 10T0.1E buffer (makes 50 mL)
   i. Add the following ingredients to a 50mL sterile falcon tube.
      1. 10µL 0.5M EDTA
      2. 500µL 1M Tris-HCl
   ii. Fill the tube with sterile, PCR water for a final volume of 50mL.
   iii. Cap tube and gently invert to mix well.
   iv. Store at room temperature.

d. 7.5M Ammonium acetate (makes 50mL)
   i. Add 28.5g ammonium acetate to a 50mL sterile falcon tube. Fill the tube with sterile, PCR water for a final volume of 50mL.
   ii. Cap tube and gently invert to mix well.
   iii. Store at room temperature.

2. Protocol for extractions

DAY 1

a. Preheat the 4M urea buffer to 60°C using a heating block.
b. For samples stored in ethanol, first use sterile forceps to remove tissue from the tube and place on a new delicate task wipe.
c. If tissues are too large, trim to size (0.5mg), making sure that all three tissue types (gill, gut, and rectum) are included.

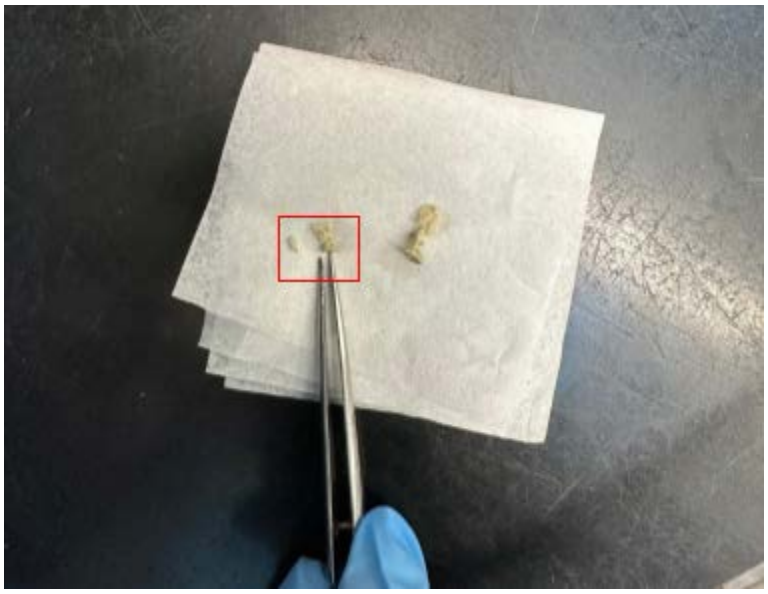


Figure 1. Size comparison of 0.5mg of gut, mantle, and gill tissue samples from eastern oysters for DNA extraction. Size of each tissue type is no larger than the tip of a fine-point forceps.

d. Fold the wipe over the tissue and press firmly to blot samples dry of ethanol. Place tissue into a new 1.5mL microcentrifuge tube and keep the lid open to allow ethanol to continue evaporating. All tools are sterilized between individuals following the steps listed below:
    i. Freshwater dip
    ii. Bleach and sand dip to remove tissue
    iii. Freshwater dip
    iv. 95% ethanol dip
    v. Ethanol is burned off using a flame (allow to cool before reusing)
e. For every 25-30 samples, include an additional sample tube for a blank or negative control. This blank will go through the extraction process and have all of the buffers but will not contain any tissue. This will help identify if any contamination is happening during extractions. This blank should have an end DNA concentration of 0 ng/uL.
f. Pipette 180µL of 4M urea buffer into each test tube.
g. Close lids and vortex each sample thoroughly.
    i. Be sure they snap fully closed before vortexing
h. Incubate samples at 60°C on a heating block for 60 minutes, pausing to vortex the samples every 15 minutes. *After 60 minutes, set the heating block to 95*°C for step “i” below.
i. While samples are incubating, fill another set of correctly labeled 1.5mL microcentrifuge tubes with:
    i. 1 µLl of 100TE buffer (to adjust the pH to 8.0)
    ii. 50 µL of ammonium acetate
    iii. 400 µL of 100% ethanol

j. After the first 60 minutes of incubation, vortex samples, then incubate samples at 95°C on the heating block for 15 minutes.

k. Vortex samples, then centrifuge at 15,000 x g for 5 minutes.

l. Add 100µL of the prepared sample to the corresponding tube containing ethanol, ammonium acetate, and 100TE buffer (**be very careful to avoid chelex beads in this step*)

m. Incubate the samples at -20°C freezer overnight.

DAY 2

n. After overnight incubation, centrifuge samples for 20 minutes at 15,000 x g.

o. Being careful not to disturb the pellet, decant liquid from the tube by pouring into a designated chemical waste container.

p. Add 200µL of ice cold 70% ethanol and flick the tube to wash the pellet.

q. Centrifuge samples at 15,000 x g for 5 minutes to re-pellet DNA.

r. Repeat the wash step 2 more times. **After the 3rd wash do not pour out ethanol. Use filter tips to pipet the ethanol out**

s. Place tubes upside down on paper towels and let air dry for approx. 30 minutes. * *all ethanol must be evaporated from the tube before continuing - may need to carefully insert a kim wipe to remove excess ethanol from sides**

t. Resuspend the pellet in 100µL of 10T0.1E buffer and flick each tube to fully dissolve the pellet.

u. At this stage, the DNA extracts can be stored in the freezer (-80°C) until further use. ***For the Triplex qPCR protocol DNA extractions are diluted to 100ng/3µL (as described in Section 9.2.3, Nanodrop)*

3. Nanodrop

a. After every set of extractions the samples should be tested for DNA concentration using the Nanodrop in Bldg 1, room 18. All samples for the Triplex qPCR assay will be adjusted to a concentration of 33.3 ng/µL of DNA.

b. Turn on the Nanodrop and set-up the run according to the instrument instructions.

c. Wipe the pedestal on both sides using a delicate task wipe.

d. Pipette 1µL of elution buffer onto the pedestal to act as a blank.

e. Wipe the pedestal on both sides using a delicate task wipe again.

f. Vortex samples to mix DNA.

g. Pipette 1µL of the desired DNA sample onto the pedestal to determine the purity and concentration of DNA. Document data, clean pedestal with a delicate task wipe, and duplicate to create 2 replicates for each sample.

As samples are run, evaluate sample quality using the following guidance:

i. A260/A280 ratio: A ratio of 1.8–2.0 indicates the sample is “pure”. A markedly low ratio indicates the sample may be contaminated with reagents from the extraction protocol. This number may also be off in samples with low concentrations <10ng/µL. If the duplicate samples have

ratio <1.8, vortex and rerun the sample. If the issue persists, consult with the project lead on how to proceed.

ii. Precision: Duplicates should be within 10ng/µL of each other. If they are off try to vortex and rerun the sample. Small bubbles, dust, etc. can affect the reading. If the issue persists, consult with the project lead on how to proceed.

h. Output Nanodrop data to a spreadsheet file and save.

i. For each sample, calculate an average DNA concentration from the duplicates.

j. Using the average sample concentration data, calculate the required volumes of the initial sample DNA (neatstock) and PCR water required to create new samples (analyte) with adjusted concentrations of 100ng/3µL in 50µL aliquots.

Calculate using the equation: $\mathbf{C_1V_1 = C_2V_2}$ where,

C_1= Concentration of neatstock (average of Nanodrop duplicates),

V_1 = Volume of neatstock (Unknown),

C_2= Concentration of analyte (100ng/3µL), and

V_2 = Total volume of analyte (50µL)

The required volume (µL) of neatstock to create the analyte sample = V_1.

The required volume (µL) of PCR water to create the analyte sample = 50-V_1.

k. To create the analyte samples, first pipette the calculated volumes of water into the pre-labeled strip tubes. Then, using filter tips, pipette the calculated volume of neatstock DNA into the corresponding strip tube.

l. Both neatstock and analyte samples can be stored in the freezer (-80°C for long-term storage, and -20°C for short-term) until further use.

4. Clean up

a. Properly dispose of any chemical waste.

b. Wash and autoclave any glassware.

c. Autoclave any biological waste and dispose of trash.

d. Sterilize bench tops and equipment with 10% bleach solution.

i. Spray benchtops with 10% bleach

ii. Let sit 5 minutes

iii. Wipe down with paper towels to dry

10 Data Quality Control

Laboratory data will be recorded on data sheets and transcribed to digital data records on the secure NOAA server within 3 business days of the date of collection and processing. This will ensure all notes are correctly updated while the events are fresh in the team's memory. Digital data files will be QC'd by a second individual alongside the data sheets to ensure all data was correctly entered within one week of data entry. Discrepancies in the data will be resolved by comparing data sheets and digital logs alongside lab notebooks that contain site and date specific information that could explain outliers in the data. A

record of the lab recorder, person entering the data, and QC completion will be logged to ensure all tasks are completed prior to final data collation. Each month after QC, data will be collated with the previous data and exploratory plots updated. This will serve as an added check for potential error in the data collection and archiving process.

All laboratory data sheets will be scanned and filed on the NOAA server in the project folder. Physical copies will also be filed for archiving at the NOAA Milford Laboratory. Purely digital files (eg. sonde data, photos, etc.) will be uploaded directly to the NOAA server which is backed up regularly.

11 References

Aranishi F, Okimoto T (2006) A simple and reliable method for DNA extraction from bivalve mantle. J Appl Genet 47(3):251-254.

END OF SOP

Appendix D – Triplex qPCR assay for detection and quantification of Dermo/MSX/SSO pathogens SOP

1 Purpose

The purpose of this document is to provide a clear methodology for Triplex qPCR to identify *Perkinsus marinus, Haplosporidium nelsoni*, and *Haplosporidium costale*, respectively, the causative agents of Dermo, MSX, and SSO diseases in oyster tissue that has been extracted for DNA.

2 Scope

This SOP applies to the Northeast Fisheries Science Center, Milford Laboratory staff performing qPCR on DNA samples from eastern oysters, *Crassostrea virginica*, collected for the Long Island Sound Study (LISS) funded project which aims to identify and quantify infections of common oyster pathogens in oyster tissue.

3 Definitions

Unknown -sample of DNA extracted from oyster tissues collected for diagnostic testing

4 Reagents/Media

- Promega GoTaq qPCr Master Mix, A6101, Promega, Madison, WI
- Probes and Primers Dermo/MSX/SSO, Integrated DNA Technologies, Coralville, Iowa. * Note supermix primers, and probes are stored in the -20°C freezer.*
    - Dermo Forward Primer: CGCCTGTGAGTATCTCTCGA
      Dermo Reverse Primer: GTTGAAGAGAAGAATCGCGTGAT
      Dermo Probe: 56-FAM/CGCAAACTCGACTGTGTTGTGGTG/3BHQ1
    - MSX/SSO Forward Primer: ACAGGTCAGTGATGCCCTTAG
      MSX/SSO Reverse Primer: TSGRGATTACCYSGCCTTC
      MSX Probe: SHEX/ TTGCACGCAACGAGTTCAACCTTGCCTG /3BHQ_1
    - SSO Probe: 5Cy5/AATGACCCAGTCAGCGGGCCGA/3BHQ_1
- Plasmid standard Curve (10 to 10^8 copies)

5 Supplies/Materials

- Nitrile gloves
- Lab coats
- PCR water: UltraPure DNase/RNase-Free Distilled Water (PCR water), 10977023, Invitrogen, USA
- Ice bins
- Crushed Ice
- 1.5mL microcentrifuge tubes PCR clean
- 2mL microcentrifuge tubes PCR clean
- Foil
- Strip tubes: USA Scientific TempAssure 0.2ml PCR 8-tube strips Cat. No. 1402-4700

- PCR Plates: USA Scientific TempPlate No-skirt 0.1ml PCR plates, white Cat. No. 1402-8590
- PCR Plate Seals: BioRad Microseal 'B' plate seals Cat. No. MSB1001
- Pipettes
- Sterile Pipette tips (Filter Tips: 1000 µL, 200µL, 50µL, 10µL, 2µL)

6 Equipment

- UV compatible hood
- Plate centrifuge/spinner
- Bio Rad CFX96 Touch Real-Time PCR machine
- Vortex
- Small centrifuge for tubes

7 Safety Precautions

Lab members will wear gloves and lab coats while performing qPCR assays. Members will follow standard laboratory procedures in the event of an accident (i.e. spilling chemicals, eye contamination, chemical burns) as well as for disposal of chemicals and any hazardous wastes. Safety Data Sheets (SDS) can be found in designated binders in each labspace.

8 Laboratory Quality Control

Lab members will use a "qPCR hood" that is compatible with UV light to sterilize the area before preparing the assay. Only the assay will be prepared in the hood. No DNA or plasmid DNA is to enter the hood to reduce contamination in the assay. Members will also wear gloves and change them frequently through various steps in the process (see below). There will also be designated areas for DNA and plasmid DNA handling. Foil will be used to cover probes and reagents that are light sensitive as well as the plate during the preparation of the assay and adding samples/standards. The assay has been thoroughly tested for sensitivity and specificity. The assay has a detection limit of 10-100 copies of plasmid DNA for *P. marinus* and *Haplosporidium spp* and 80-100% concordance between singleplex qPCR for *P.marinus* and *H. nelsoni*, and gel-based PCR for *H. costale* for detection. The overall concordance between histology and qPCR was 54, 57, and 87% for *P.marinus*, *H. nelsoni* and *H. costale*. Discordance is due to increased sensitivity of the qPCR assay compared to histology which can have false negatives (Piesz et al. 2022). The ideal efficiency % for this assay is 95+% with an R^2 value close to 1.

9 Preparation

1. At least one day prior to running the assay, examine the area to make sure the lab is ready for molecular work.
2. Biological trash bins should also be emptied, autoclaved, and properly disposed of. Bins should not exceed 75% full.
3. Sign up or schedule time to use any machines (i.e. CFX96 real-time qPCR machine) if necessary. Allow enough time for preparing the assay before run time. For example, scheduling a run at 11am with prep time from 9:30-11am.
4. Check that all equipment is running and calibrated such as the UV light in the hood, centrifuges, plate spinners, and the CFX96 real-time qPCR machine.

5. All laboratory stations should be set up and cleared of unnecessary equipment for easy work flow.
6. Probes and Primers
   a. Primers and probes are rehydrated to 100µM stock concentrations (primer stock) and diluted into working stocks of 10µM (working stock).
      i. Rehydrate primers according to the tube. If the tube from IDT reads '26.3nmol' add 263µL PCR water. This is the primer stock.
      ii. To make working stocks, add 90µL PCR water to 10µL of primer stock (final concentration 10µM).
7. Complete the plate template sheet and double check the automatically generated calculations for the master mix and sample volumes. A list of all required reagents, samples, and their volumes per reaction (plate well) are provided in **Table 1.** Print a copy of the plate template sheet to refer to while working.
8. Check the supplies and materials list and take inventory. Make sure there are enough reagents available for the scheduled assay preparation. Check that all primers and probes have been diluted into working stocks of 10µM. If they are not, prepare primers and probes in desired quantities.

Table 1. Required volumes of reagents and DNA samples per reaction (PCR plate, 96 wells) for the Triplex qPCR assay of *P. marinus* (Dermo), *H. nelsoni* (MSX), and *H. costale* (SSO).

	Standards	**Unknowns**	**No Template Control**
Master Mix (MM)*	10 µl Supermix** + 0.6 µl Dermo Forward Primer + 0.6 µl Dermo Reverse Primer + 0.9 µl MSX/SSO Forward Primer + 0.9 µl MSX/SSO Reverse Primer + 0.15 µl Dermo Probe + 0.15 µl MSX Probe + 0.15 µl SSO Probe + 3.55 µl PCR Water	10 µl Supermix** + 0.6 µl Dermo Forward Primer + 0.6 µl Dermo Reverse Primer + 0.9 µl MSX/SSO Forward Primer + 0.9 µl MSX/SSO Reverse Primer + 0.15 µl Dermo Probe + 0.15 µl MSX Probe + 0.15 µl SSO Probe + 3.55 µl PCR Water	10 µl Supermix** + 0.6 µl Dermo Forward Primer + 0.6 µl Dermo Reverse Primer + 0.9 µl MSX/SSO Forward Primer + 0.9 µl MSX/SSO Reverse Primer + 0.15 µl Dermo Probe + 0.15 µl MSX Probe + 0.15 µl SSO Probe + 3.55 µl PCR Water
Total Volume	17µL	17µL	17µL
DNA Standard/ Sample	1µL Dermo DNA + 1µL MSX DNA + 1µL SSO DNA	3µL of unknown sample DNA	3µL of PCR water
Total Reaction Volume (MM + DNA)	20µL	20µL	20µL

*The Master Mix will be made to final concentrations of 30nM Dermo primers, 450nM MSX/SSO Primers, and 75nM of all Probes per reaction.

** Supermix = Promega GoTaq qPCR MasterMix

10 Procedures

1. Please see **Appendix A** & **C** for sample preparation prior to running qPCR assay.
2. Hood/Workstation set up
   a. Obtain a 2mL microcentrifuge tube for master mix, plate seal, 96 well PCR plate, and an ice bin and put supplies into the qPCR hood. Turn on the UV light on the PCR hood and leave on for 15 minutes to decontaminate the area. Turn off UV light before proceeding to the next step.
   b. Add ice to the ice bin that is in the hood for reagents.
   c. Obtain two additional ice bins for unknowns and the DNA standards (separate bins), and add ice to those bins. These do not go into the hood. Place bins on a bench outside of the hood.
   d. Change gloves.
   e. Get your unknown sample tubes of extracted DNA out of the freezer (-20℃ or -80℃). Allow the samples to thaw and then place them into the ice bin on the bench in order as listed on the plate template.
   f. Get your standards (in 4℃ always) and put them directly on ice in order of dilution as listed on the plate template.
   g. Change gloves.
   h. Obtain reagents for the PCR reaction and place them inside the hood (not on ice).
      i. 10µM working stock of primers and probes. *Probes are always covered in foil to prevent degradation from UV exposure.*
      ii. Supermix (Promega GoTaq qPCR Master Mix)
      iii. PCR water
3. Create the master mix
   a. Pipette the required volume of reagents, primers, and probes based on the calculations on your plate set up sheet (**Section 9.7**). Refer to **Table 1** for required volumes per reaction. ** Return reagents immediately to fridge or freezer after use **
   b. Vortex the tube of master mix to combine and briefly spin down using a bench top centrifuge to remove any liquid from the cap.
4. Setting up plate
   a. Add 17µL of master mix to each well on the plate according to the plate set-up sheet. Notes to reduce potential contamination and error:
      i. Work from left to right on the plate.
      ii. To reduce pipetting error, wipe the side of the pipette tip on the inside of the mastermix tube as you take out the aliquot.
      iii. Do all duplicate columns one after each other to ensure similar mastermix in each.
      iv. Cover each duplicate column (16 wells) with foil as you go.
      v. Keep everything foiled as long as possible.

b. For no template controls, add 3µl of PCR water to your control wells. These are typically the last two wells on the plate (refer to plate set-up sheet). Cover with strip tube tops to reduce contamination.

c. Unknown samples – In reverse order on your plate, add 3µl of unknown sample DNA into the appropriate wells (refer to plate set-up sheet). Duplicate reactions will be run for each unknown sample.

   i. Before pipetting the unknown DNA, finger flick the tube to mix.

   ii. Pipette up and down 10 times to keep samples homogenized.

   iii. As you add the sample to the well, pipette up and down into the mix.

   iv. Once DNA is added, put the samples back into the ice bin.

   v. Keep foil over wells as you go - covering before and after DNA is added.

   vi. Return samples immediately to the freezer after adding to the plate.

d. Standards – plasmid DNA standards for each disease at 10-fold dilutions series from 10^8 copies to 10 copies per reaction are used for this assay.

   i. Work from the lowest, most dilute standard to the highest, most concentrated dilutions (10 copies up to 10^8 copies)

   ii. Vortex the tube of standard DNA for 10 seconds (use a timer for consistency) and spin down briefly using a bench top centrifuge to remove liquid from the cap. Add 1µL of the appropriate dilution into the well with master mix in triplicate. Add standard to all replicates before moving onto the next dilution. Make sure to cover wells with foil as you go. Put tubes back into the ice bin when finished.

   iii. Return tubes of standards to the fridge immediately after adding to the plate.

   iv. Note – Plasmid sizes for DNA standards:

      1. Dermo plasmid size = 90bp insert + 3956bp vector = 4046bp
      2. MSX plasmid size = 725bp insert + 3956bp vector = 4681bp
      3. SSO plasmid size = 718bp insert + 3956bp vector = 4674bp

5. Change gloves

   a. To seal the plate, first remove the backing from the plate seal. Place the sticky side down onto the plate from left to right covering all the wells. Once you get to the negative controls, remove the strip tube tops and cover with seal. Make sure all edges are sealed by applying pressure.

   b. Take the sealed plate to a centrifuge with a plate attachment (cover the plate with aluminum foil while transporting). Spin the plate at 3500 rpm for 3 min to remove all air bubbles.

6. Next, it is time to set up the Triplex qPCR reaction cycle on the Bio Rad CFX96 Touch Real-Time PCR machine. Duration and temperature of the cycles are as follows:

   a. 95℃ for 60 seconds

   b. 40 cycles of: 95℃ for 15 seconds and 60℃ for 30 seconds

   c. 4℃ forever

7. Configure the software for the assay by doing the following:

a. Open Bio-Rad CFX Maestro Software
b. Choose “User-defined” on selected run type options, then hit OK
c. Choose your thermal cycling protocol to run
    i. If setting up a new run, click “Create New”
        1. Insert temperature steps as needed
        2. Adjust temperature and time per step
        3. Add Go To step (go to step 2, x39)
        4. Insert Melt Curve if needed after cycling
        5. Last step should always be 4℃
    ii. Or click “select existing” to select a protocol from existing files.
d. Set-up your Plate:
    i. Click “Create New”
    ii. Click “Select fluorophores” button on right menu bar:
        1. FAM – Dermo, *Perkinsus marinus*
        2. HEX – MSX, *Haplosporidium nelsoni*
        3. Cy5 – SSO, *Haplosporidium costale*
    iii. Click “OK”
    iv. Setting up Standards:
        1. Highlight the wells for your “standards”
            a. 2 columns (samples in duplicate)
        2. Go to “sample type” on right drop down menu bar
            a. Select “standard”
        3. “Load the fluorophores” by checking each box
        4. Click “Technical Replicates”
            a. Selected replicate size= 2
            b. Starting # 1
            c. Horizontal series
            d. Click “Apply” (should show standard 1-8 with 2 replicated each in the appropriate well on the “plate”)
        5. Click “Dilution Series”
            a. Change the starting concentrations to 1.00E+08 (10^8)
            b. Make sure the dilution factor is 10
            c. Click “apply” (should show dilutions for the standards in each appropriate well on the “plate”)
    v. Setting up Unknowns:
        1. Highlight the wells for your “unknowns” in groups of 8
            a. 2 columns (samples in duplicate)

2. Go to “sample type” on right drop down menu bar
    a. Select “unknown”
3. “Load the fluorophores” by checking each box
4. Click “Technical Replicates”
    a. Selected replicate size= 2
    b. Starting # 1
    c. Horizontal series
    d. Click “Apply” (should show unknowns 1–8 with 2 replicated each in the appropriate well on the “plate”)

vi. Setting up the non-template control:
1. Highlight the wells for your “no template control”
    a. Only will have 2 (duplicates)

vii. Go to “sample type” on right drop down menu bar
1. Select “no template control”

viii. “Load the fluorophores” by checking each box

ix. Click “Technical Replicates”
1. Click “replicates” box
2. Starting # 1

e. Check plate settings in the tab on the top row in the plate editor taskbar.
    i. Check that the plate size is set to “96 wells”
    ii. Change plate type from “Clear” to “White”
    iii. Change “Units” to “copy numbers”
    iv. Click “OK”
    v. Save the plate in the designated project folder.
f. When the plate and software set-up are complete, the plate can be put into the thermocycler.
    i. Hit “next” or “start run”
    ii. Click “start run” tab
        1. Will be prompted to save run
            a. Save same as plate file but remove ‘.pltd’

8. Data reporting

a. After the run is complete, record on the plate template sheet the efficiency % and R^2 values. Ideally an efficiency of 95+% and an R^2 close to 1 identifies if the reaction was successful and there was minimal pipette error.
b. Next, identify that the standard curves are of quality to compare the data. This would include low variation between replicates and a close estimate to the goal concentration.

c. Read the melt curves and amplification plots to make sure there was proper amplification during the temperature cycles.

d. Note any discrepancies in the samples on the plate template sheet and place into the appropriate laboratory notebook.

e. Lastly, pull the data file from the program that provides the unknown quantifications for further analysis and calculation of copies/mg of tissue.This digital copy will be stored in a repository in the data management system.

f. Technical replicates should have ≤2.0% difference in CT reads. Failure to meet this DQAO will initiate a second qPCR run of the sample, but in triplicate.

g. If there is high variation (identified on the machine ≤ 2% C(t)) between samples, the efficiency % is less than a minimum of 92%, or the R^2 is not close to 1 (i.e., 0.899 and below) the assay or specific samples should be rerun on a new plate. If the assay continues to have low values or high deviation check the calculations for the mastermix and consult with the laboratory supervisor.

h. For this assay, the detection limit for the assay is 10 copies per 1µl or sample. If the DNA being detected is lower than this number it can be considered “Target DNA not detectable” or no prevalence in the sample. Values higher than 10 copies per sample (maximum 10^8) are indicated to be positive or have detectable target DNA.

9. Clean up

a. Make sure all reagents, samples, and plasmid standard curves have been returned to their designated fridge or freezer.

b. Empty ice bucket in the hood and return the empty bucket to the hood. Turn on UV for 15 minutes.

c. Empty ice buckets on the benches that had DNA or plasmid DNA. Wipe them out with bleach (10%).

d. Bleach (10%) benches where DNA or plasmid DNA was opened/used.

e. Make sure all other supplies (plates, seals, pipettes, etc.) have been put away in their designated locations.

11 Data Quality Control

Laboratory data will be recorded on data sheets and transcribed to digital data records on the secure NOAA server within 3 business days of the date of collection and processing. This will ensure all notes are correctly updated while the events are fresh in the team’s memory. Digital data files will be QC’d by a second individual alongside the data sheets to ensure all data was correctly entered within one week of data entry. Discrepancies in the data will be resolved by comparing data sheets and digital logs alongside lab notebooks that contain site and date specific information that could explain outliers in the data. A record of the lab recorder, person entering the data, and QC completion will be logged to ensure all tasks are completed prior to final data collation. Each month after QC, data will be collated with the previous data and exploratory plots updated. This will serve as an added check for potential error in the data collection and archiving process.

All laboratory data sheets will be scanned and filed on the NOAA server in the project folder. Physical copies will also be filed for archiving at the NOAA Milford Laboratory. Purely digital files (eg. sonde data, photos, etc.) will be uploaded directly to the NOAA server which is backed up regularly.

12 References

Piesz, J. L., Scro, A. K., Corbett, R., Lundgren, K. M., Smolowitz, R., & Gomez-Chiarri, M. (2022). Development of a multiplex qPCR for the quantification of three protozoan parasites of the eastern oyster Crassostrea virginica. Diseases of Aquatic Organisms, 151, 111-121.

END OF SOP

Appendix E – Davidson's Fixative and Histology Slide Preparation SOP

1 Purpose

The purpose of this SOP is to provide concise guidance and standard methodology on how to make Davidson's fixative and perform histology slide preparation after tissue fixation for microscopy to observe gonadal condition determination and disease.

2 Scope

This SOP is pertaining to the Long Island Sound Study (LISS) Project where preserved oyster tissue will be processed using histological methods.

3 Definitions/Acronyms

NaCl- sodium chloride
ETOH - Ethanol
DI water - DeIonized Water
Embedding - the process of placing tissue in a firm medium to keep it intact when cutting sections for histological examination.

4 Reagents/Media

- Glycerin
- Formaldehyde
- ETOH
- Glacial Acetic Acid
- Xylene
- Harris' Hematoxylin
- Concentrated HC1 sp. gr. 1.19
- Lithium Bromide
- Eosin Y

5 Supplies/Materials

- Tissue cassettes
- Scissors
- Beaker/Graduated cylinder
- Paraffin
- Paraffin scraper
- Forceps/Probes/Camel-hair brush
- Metal trays
- Ice bath
- Filing trays
- Thermometer
- Diamond pen
- Microscope slides
- Coverslips
- Distilled water
- Paper towels
- Gloves
- Lab coats
- Pipette + tips
- Disposable blades (for microtome)

- Stain containers
- Slide dippers
- Timers
- Permount

6 Equipment

- Chemical fume hood
- Leica TP 1020 - Automatic Tissue processor
- Tissue-Tek TEC 5 Tissue Embedding Console System
- Microtome
- Scale
- Heat plate
- Water bath
- Drying oven
- Paraffin Dispenser
- Refrigerator

7 Safety Precautions

Laboratory members will wear gloves and protective eyewear when using chemicals. Members will follow standard laboratory procedures in the event of an accident (i.e. spilling chemicals, eye contamination, chemical burns) as well as for disposal of chemicals and any hazardous wastes. Chemicals will be clearly labeled and kept under a fume hood if necessary. Disposable pipette materials will be used when working with chemicals. Lab members will have knowledge of the location of the nearest first-aid kit. Safety Data Sheets (SDS) can be found in designated binders in each labspace.

8 Laboratory Quality Control

Laboratory members will wear gloves and lab coats to reduce the risk of contamination. All equipment will be sterilized before and after use, as well as in between individual organisms, where appropriate. Tubes and slides will be pre-labeled prior to histology to streamline the process and prevent samples from being incorrectly identified. There will be designated stations/lab areas for each process. Guidelines for histology and other steps in the process will be discussed between members prior to starting to ensure uniformity.

9 Preparation

1. Preparation of Tissue- See **Appendix A**.
2. Pre-label all microscope slides with pencil on the frosted portion of the slide
    a. Label should include Oyster ID #, Site, Collection Date, Stain.

10 Procedures

1. Davidson's Fixative
   *** Under a chemical fume hood ***
    a. To create a stock solution of Davidson's Fixative, add the listed ingredients to a prelabeled 1L plastic bottle with a screw cap. This should be done ahead of sampling days.
        i. 100 ml Glycerin
        ii. 200ml Formaldehyde
        iii. 300 ml Ethanol (95%)

iv. 20 g NaCl in 300 ml Deionized water

b. Rinse graduated cylinder with water under the fume hood then leave in the fume hood to further exhaust fumes and avoid accidental inhalation of fumes. DO NOT take directly to a sink outside of the hood to rinse chemicals.
c. When ready to cut oysters, make a working solution of Davidson's Fixative by adding the following ingredients to a formalin-safe container intended for sample cassettes.
    i. 50mL Glacial Acetic Acid
    ii. 450ml of Davidson's Fixative.
    iii. Note: To achieve the desired fixative:tissue volume ratio of 15:1 to 20:1, for sample sizes of 50 oyster use 630ml of Davidson's fixative and 70 ml glacial acetic acid.
d. Cassettes with tissue sections should be added to the container of Davidson's Fixative (working solution) under the hood to avoid fume inhalation (See **Appendix A** for details on this step)
e. After adding all sample cassettes to the fixative, store for 24-48 hrs at 4℃ (standard refrigeration).
f. Post-refrigeration, dispose of the fixative into a designated chemical waste container under the fume hood using the designated Davidison's Fixative funnel.
g. Add 70% ethanol to cover cassettes and store at 4℃ (standard refrigeration).
    i. Label the bottle with the sample details, change in media, date, and initials
        1. Sample details (type, number), 70% EtOH, MM/DD/YYYY, initials
    ii. Tissues are now stable in this condition and can be stored for several months.

2. Tissue Processing and Embedding (Howard et al. 2004) - This portion of the protocol will take place at the CT DABA Histology Lab (contact: Lydia Bienlien, Shellfish Pathologist, lydia.bienlien@ct.gov)
    a. After fixation and ethanol rinse, tissue will be processed on a Leica TP 1020 - Automatic Tissue processor located at the CT DABA laboratory following this 12 step routine procedure using EtOH, Xylene, and paraffin.
        i. Paraffin with a melting point of 56.6°C is the most satisfactory for embedding.

Step	Action	Reagent	Time
1	Dehydrate	95% EtOH	1hr
2	Dehydrate	100% EtOH	1hr
3	Dehydrate	100% EtOH	1hr
4	Dehydrate	100% EtOH	1hr
5	Dehydrate	100% EtOH	1hr
6	Dehydrate	100% EtOH	1hr
7	Dehydrate	100% EtOH	1hr

8	Cleaning	Xylene	1.5hr
9	Cleaning	Xylene	1.5hr
10	Cleaning	Xylene	1.5hr
11	Infiltration	Paraffin	2hr
12	Infiltration	Paraffin	2hr

b. When the automatic processor has completed this cycle, transfer all slides into a transport bin and take to the embedding station.
c. Prior to embedment of tissue, a release dip can be used to coat the molds made up of 95 ml of 95% ethanol and 5 ml of Glycerin.
d. Samples will be embedded using a Tissue-Tek TEC 5 Tissue Embedding Console System following standard procedures described below.
e. Place several tissue cassettes in a small paraffin bath.
f. Using heated forceps, remove identification labels from corresponding tissue in the tray.
g. Find the matching embedding ring and choose a mold of the appropriate size. Tissue cassettes allow the tissues to be transferred into molds. and the labeled cassette becomes the top for each paraffin block.
h. Hold the mold by its handles and cover the bottom of the mold with heated paraffin from the dispenser (no more than ¼ full).
i. Using heated forceps, turn the tissue over and gently squeeze to release any trapped air bubbles. Quickly orient the tissue as desired.
j. Place the cassette onto the base mold with the correct identification number and hold it in place with your index finger.
k. Fill the mold to the top with paraffin and place onto the cooling unit of the embedding center or into a shallow container of ice water for approximately 10 minutes. This step should be done as quickly as possible.
l. After the block has cooled, use gentle pressure to remove it from the mold for trimming and sectioning.
m. Place the block in a filing tray and refrigerate prior to sectioning.

3. Sectioning General (Howard et al. 2004)
    a. Set up a water bath for step ‘i’. Temperature should be ~42°C. (Water bath should be changed daily and kept clean and clear of air bubbles.)
    b. Align firm blocks of paraffin with tissue in consecutive order according to their identification code.
    c. Rough cut or trim the paraffin at room temperature and precool to 5°C before sectioning. A warm block will not section properly.
    d. After precooling, place the blocks into an ice bath.
    e. Carefully mount the cassette in the microtome vise on the tissue processor positioning the tissue gills or identification number to the right. Slightly angle the head of the microtome if necessary to expose all of the tissue without cutting the block too thin.
    f. Rotate the block so that its bottom surface is parallel to the edge of the knife. Make sure the microtome knife is angled based on the type of blade being used.
    g. Trim the block of paraffin until the tissue is fully exposed.

h. Set the microtome for section thickness - 5-6 µm - and begin cutting the sections. Make sure to have continual even strokes when cutting to create a ribbon. Ribbons are preferred to create uniform thickness.
i. Gently lift the ribbon using forceps and a small paint brush and float the ribbon of sections on the heated water bath.
j. Before mounting on a slide, make sure the slides are labeled properly to ensure identification of the tissue (Same label ID as on the cassette). Scratch the code number of each tissue on the reverse side of a frosted, coated slide using a diamond pen if possible.
k. Separate the desired tissue section and dip the prepared and coded slide, frosted side up, into the water bath under the tissue section. Guide the section into place with a small camel-hair brush (paint brush).
l. Gently raise the slide from the waterbath and cut excess paraffin from each side. Make sure the tissue is centered using a probe or brush. Leave at least ⅛ inch clearance at the end of the slide.
m. Place the slides in a rack to drain excess water and then into a drying oven at 42°C for about 12 hrs or until completely dry.

NOTE: The temperature of the water bath and oven should not exceed 42°C for tissues embedded in paraffin. If the tissue begins to melt on the slides it can cause distortion of cells in the tissue.

4. Staining- Harris' Hematoxylin and Eosin$_2$ (HHE$_2$) (Howard et al. 2004)
   Under a chemical fume hood
   a. Remove dried slides from the oven and place them in the staining holder with the frosted end up and facing forward. Make sure they are in order.
   b. Samples will be hand stained using Harris Hematoxylin and Eosin Y-Phloxine B by moving the staining holder with slides through a series of staining baths using the following procedure:

Step	Action	Reagent	Time
1	Deparaffinize	Xylene	3min
2	Deparaffinize	Xylene	2min
3	Hydrate	100% Ethanol	15 dips
4	Hydrate	100% Ethanol	15 dips
5	Hydrate	95% Ethanol	15 dips
6	Hydrate	95% Ethanol	15 dips
7	Hydrate	running tap water	3min
8	Stain	Harris Hematoxylin	8min
9	Stain	Running tap water	2min

10	Differentiate Hematoxylin	acid EtOH (0.3 HCl in 200ml 70% EtOH)	1.5min
11	Differentiate Hematoxylin	running tap water	5min
12	Blue-ing	NaHCO2 (5g in 200ml DI water)	1.5min
13	Blue-ing	Running tap water	5min
14	Counter Stain	Eosin Y-Phloxine B	3min
15	Differentiate eosin and dehydrate	95% EtOH	6 dips
16	Differentiate eosin and dehydrate	95% EtOH	6 dips
17	Differentiate eosin and dehydrate	100% EtOH	10 dips
18	Differentiate eosin and dehydrate	100% EtOH	10 dips
19	Differentiate eosin and dehydrate	Xylene	3min
20	Differentiate eosin and dehydrate	Xylene	5min
21	Differentiate eosin and dehydrate	Xylene	5min
22	Mount	Xylene	5min - coverslip with Permount

5. Coverslipping
    a. With the frosted, coded side of the slide facing up, take a Kim wipe and remove excess xylene from the front and back of the slides, without touching the tissue sections.
    b. Place one drop of mounting fluid on the tissue on the slide.
    c. Choose the proper coverslip, place the edge of the cover slip at an angle to the bottom edge of the slide, and gradually lower onto the slide so the mounting fluid flows evenly from bottom to top. Do not drop the cover slip onto the slide to avoid bubbles.

d. Blot edges of slide on a paper towel and examine for occasional bubbles. If present, gently lead the bubble to the edge, out from under the coverslip using a probe. Proceed with care to avoid damage to the tissue.
e. Place the slide on a tray with proper labels (code, stain, date) and place in a drying oven set at 38-42℃ for 2-5 days to allow the mounting medium to harden.
f. After hardening of the medium, clean,touch up labels and file slides. Removed excess mounting medium.

6. Slide review and interpretation
   a. *See* **Appendix F** - *“* Histological evaluation of *Perkinsus marinus* and *Haplosporidium spp.” SOP*
   b. See **Appendix G** -“Histological evaluation of oyster reproductive condition” SOP.

11 Data Quality Control

Laboratory data will be recorded on data sheets and transcribed to digital data records on the secure NOAA server within 3 business days of the date of collection and processing. This will ensure all notes are correctly updated while the events are fresh in the team’s memory. Digital data files will be QC’d by a second individual alongside the data sheets to ensure all data was correctly entered within one week of data entry. Discrepancies in the data will be resolved by comparing data sheets and digital logs alongside lab notebooks that contain site and date specific information that could explain outliers in the data. A record of the lab recorder, person entering the data, and QC completion will be logged to ensure all tasks are completed prior to final data collation. Each month after QC, data will be collated with the previous data and exploratory plots updated. This will serve as an added check for potential error in the data collection and archiving process.

All laboratory data sheets will be scanned and filed on the NOAA server in the project folder. Physical copies will also be filed for archiving at the NOAA Milford Laboratory. Purely digital files (eg. sonde data, photos, etc.) will be uploaded directly to the NOAA server which is backed up regularly.

12 References

Howard, D.W., E.J. Lewis, B.J. Keller, and C.S. Smith. 2004. Histology Techniques for marine bivalve mollusks and crustaceans. NOAA Technical Memorandum NOS NCCOS 5 218 pp.

European Union Reference Laboratory. 2009. Diagnosis by histopathology of *Perkinsus* sp. in molluscs. 2nd edition.

Carnegie, R. B. Histological Observation of *Perkinsus marinus*. Virginia Institute of Marine Science. https://www.eurl-mollusc.eu/content/download/45648/644727/file/

END OF SOP

Appendix F – Histological evaluation of *Perkinsus marinus* and *Haplosporidium spp*. SOP

1 Purpose

The purpose of this document is to provide concise guidance and standard methodology on how to diagnose and quantify the magnitude of *Perkinsus marinus* and *Haplosporidium* spp. infections of eastern oysters using histological techniques.

2 Scope

This SOP applies to Northeast Fisheries Science Center, Milford Laboratory staff who will be processing eastern oyster (*Crassostrea virginica*) tissue samples collected for the Long Island Sound Study (LISS) funded project to assess *Haplosporidium spp*.and *Perkinsus marinus* prevalence and infection intensity.

3 Abbreviations

H&E staining - Harris' Hematoxylin and Eosin staining

4 Supplies/Materials

- Gloves
- Premade histology slides
- Reporting materials

5 Equipment

- Compound microscope

6 Safety Precautions

Lab members will wear gloves to protect themselves and the histology slides being viewed. Slides will be handled with care to avoid any broken glass. If the slides are broken, lab safety procedures will be taken to ensure proper clean up and broken slides discarded in the sharps container. All lab members will be aware of the nearest first aid kit. Microscopes will remain on the bench tops.

7 Laboratory Quality Control

Multiple lab members will be analyzing the slides for diagnosis and scoring of *Haplosporidium* spp.and *Perkinsus marinus* infections at the same time to make sure the same methods are being used and results are being reported in the same manner. Slides will be analyzed in subsets over time to ensure accuracy of scoring.

8 Preparation

1. At least one day prior to viewing slides, examine the lab space to make sure the area is ready for the process.
2. Make sure microscopes are running properly.

3. Make sure all slides have been prepped, organized, and ready to view. See **Appendix A** and **Appendix E** for methods to prepare oyster tissues for histological preservation and slide preparation.
4. Create a list of slides/samples that will be viewed.
5. All data collection sheets should be organized, dated, and printed or electronically available for data recording.

9 Procedures

1. Identification of *Haplosporidium* spp.

   a. ***H. nelsoni***

   H. nelsoni is usually systemic spreading to all tissues through haemolymph sinuses, however early infections are localized to the digestive gland and intestines and to the gills. Multi-nucleated eosinophilic plasmodia (4 to 50 µm) can be seen outside of cells in connective tissue of all these organs. Sporocysts (20-50 µm in diameter) and spores (4-6 by 5-8 µm) with an operculum occur only in the epithelium of the digestive tubules of *Crassostrea virginica*. Plasmodia of *H. nelsoni* are not distinguishable from *H. costale* plasmodia except when spores are visible. (EURL 2009)

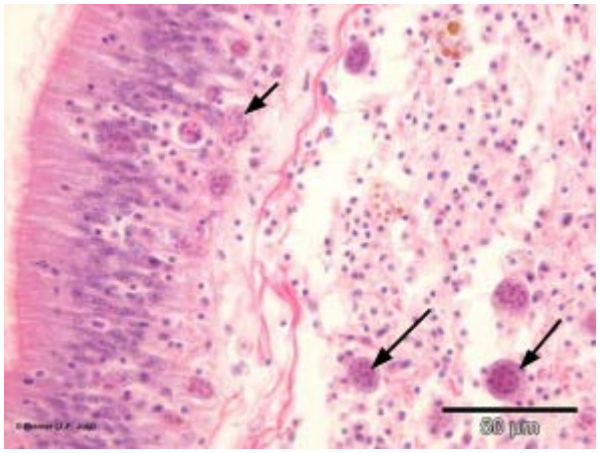


Figure 1 : Plasmodia of *Haplosporidium nelsoni* in connective tissue and intestine epithelium of *Crassostrea virginica*, H&E staining. Image from EURL Diagnosis by histopathology of *Haplosporidium sp*. in oysters.

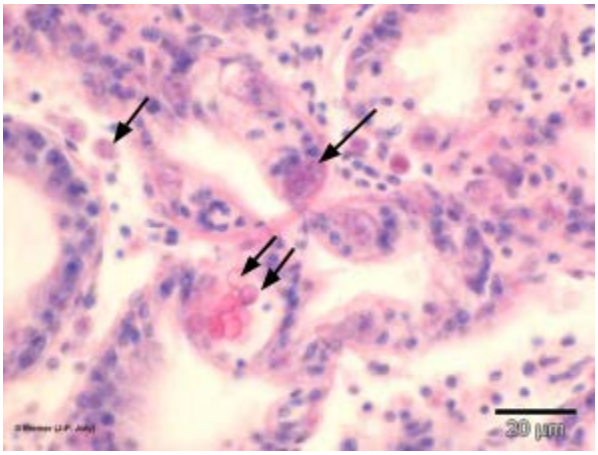


Figure 2 . Plasmodia (arrows) and spores (double arrow) of *Haplosporidium nelsoni* in connective tissue and digestive diverticula epithelium of *Crassostrea virginica*. H&E staining. Image from EURL Diagnosis by histopathology of *Haplosporidium sp*. in oysters.

b. ***H. costale***

Multinucleate plasmodium (~ 10 µm in diameter) can be observed in the connective tissue. Plasmodia develop into sporocysts with spore walls forming around the nuclei. The spores have an operculum that is smaller than *H. nelsoni* (2.6 µm by 3.1 µm). Unlike *H. nelsoni,* sporulation occurs throughout the connective tissue of the digestive gland, mantle and gonads, but not in the epithelia of the digestive tubules.(EURL 2009)

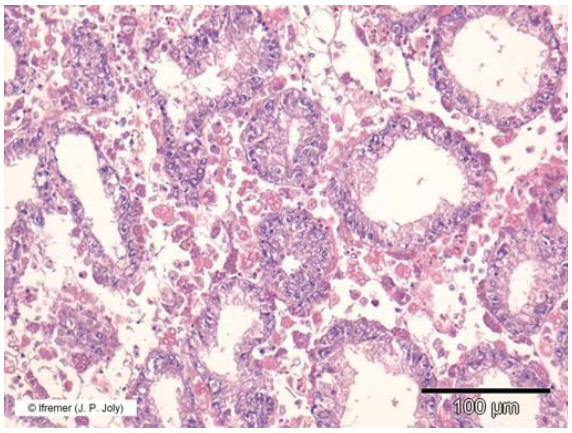


Figure 3 . Digestive gland of a *Crassostrea virginica* heavily infected by *Haplosporidium costale* with many plasmodia and sporocysts in the partially destructed connective tissue, H&E staining.Image from EURL Diagnosis by histopathology of *Haplosporidium sp*. in oysters.

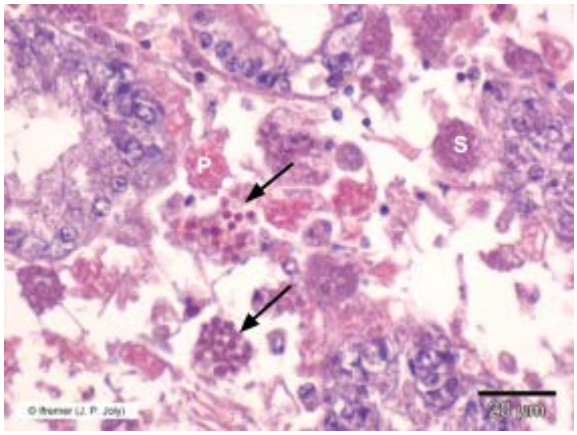


Figure 4 . Higher magnification of figure 3. Note the plasmodia (P), the sporocysts (S) and the spores (arrows) in the connective tissue, H&E staining.Image from EURL Diagnosis by histopathology of *Haplosporidium sp*. in oysters.

2. Identification of ***P. marinus***
   A positive infection is indicated by the occurrence of spherical cells about 2–15 µm in diameter, with a large vacuole and an eccentrically displaced nucleus. *P. marinus* cells stain basophilic and are often phagocytosed by host haemocytes. (WOAH, 2021). Parasites may be found in connective tissue of all organs, and in severe infections histological presentation may consist of of general invasive infiltration of haemocytes, including phagocytosis of parasite stages, disseminated in the connective tissue surrounding the epithelia of the digestive gland, gonad and mantle (Cáceres-Martínez et al. 2008).

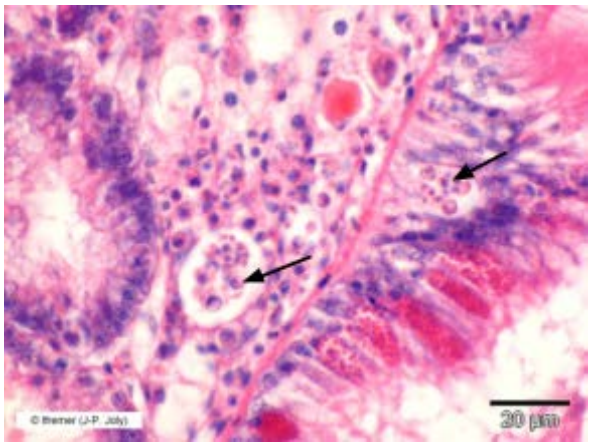


Figure 5. *Perkinsus marinus* cells in the connective tissue of the digestive gland and in the gut epithelium of an infected *Crassostrea virginica* oyster, H&E staining. Image from Ifremer Diagnosis by histopathology of *Perkinsus sp*. in molluscs, European Union Reference Laboratory.

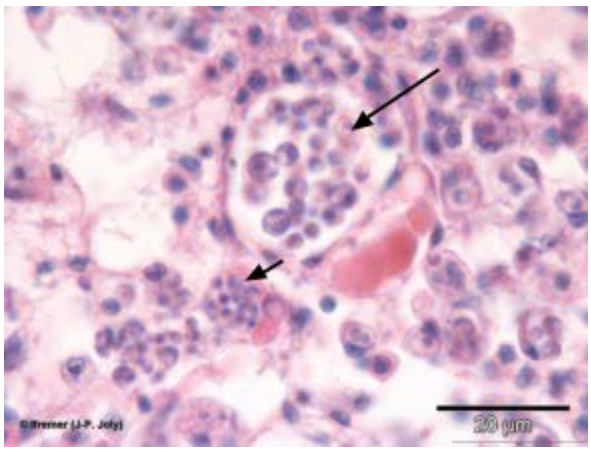


Figure 6. Different stages of *Perkinsus marinus* trophozoites in the connective tissue of the digestive gland *(Crassostrea virginica)*, H&E staining. Image from Ifremer Diagnosis by histopathology of *Perkinsus sp*. in molluscs, European Union Reference Laboratory.

3. Assigning infection intensity scores

    a. Examine prepared histological slides microscopically at 400x magnification. To assign a numerical and corresponding descriptive infection intensity, use the scoring criteria outlined in Table 1 for *Haplosporidium spp*. and in Table 2 for *Perkinsus marinus*. *Note that tissue tropism is an intrinsic part of this rating system.

Table 1. Infection intensity scoring method of *Haplosporidium spp.* as described in Carnegie and Burreson 2011.

Infection intensity	Numerical Value	Description
N (Negative)	0	No parasites observed in entire section
R (Rare)	1	1-10 cells in entire section
L (Light)	2	>10 cells observed, but focal in distribution or systemic with only 1-2 cells per field at 400x magnification
M (Moderate)	3	Systemic infection with 3-4 cells observed per field at 400x magnification
H (Heavy0	4	systemic infections with cells very abundant in connective tissues of all organs

4. Results reporting

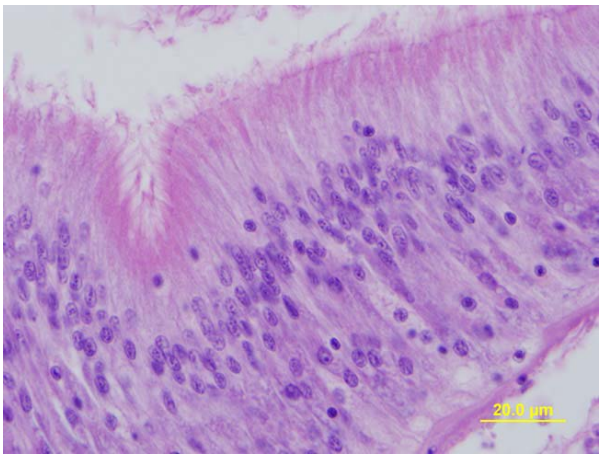
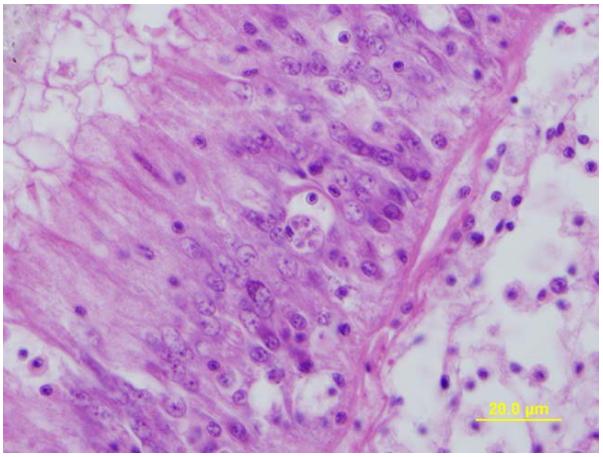
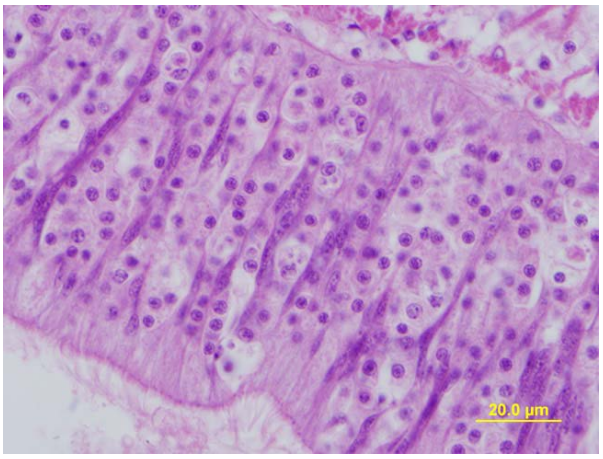
    a. After reviewing all slides, calculate the population prevalence and intensity level of *Perkinsus marinus* and *Haplosporidium spp*. infections in a given sample using the following calculations:

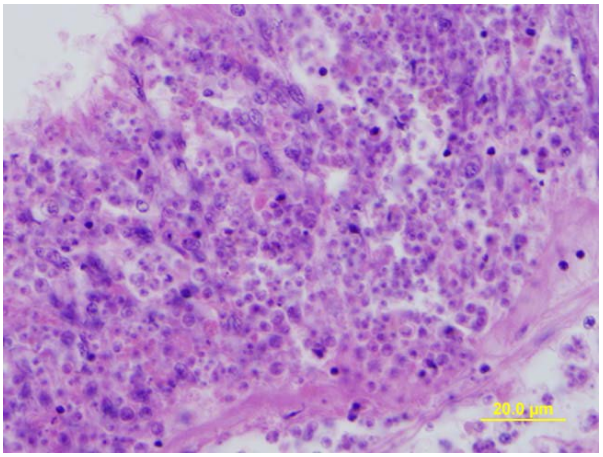
        i. $Prevalence\ (\%) = \frac{\#\ of\ infected\ individuals\ per\ sample}{total\ \#\ of\ individuals\ collected\ per\ sample}$

        ii. $Intensity = \frac{Sum\ of\ intensity\ score}{\#\ of\ infected\ individuals\ per\ sample}$

        iii. $Weighted\ Prevalence = \frac{Sum\ of\ intensity\ score}{total\ \#\ of\ individuals\ collected\ per\ sample}$

Table 2. Visualizations of histological diagnosis of *P. marinus* infection intensity as described in Mann et al., 2014. Images by Ryan Carnegie from the Virginia Institute of Marine Science.

Infection Intensity	Numerical Value	Description	Example image
Negative	0	No parasites observed in entire section	
Rare	0.5	1-10 cells or clusters of cells	
Light	1	11-30 cells or clusters of cells	
Light-Moderate	2	31-49 cells or clusters of cells	
Moderate	3	50 or more clusters of cells representing significant digestive epithelial colonization but with few cells obvious in the rest of the visceral mass	

Moderate - Heavy	4	P. marinus is clearly colonizing hemolymph spaces of the connective tissues but not to a great degree	
Heavy	5	Abundant parasites in the digestive epithelia and throughout the other tissues and organs of the host.	

5. Clean up
    a. Wipe down all surfaces.
    b. If immersion oil was used, clean all microscope lenses and wipe down the stage.

10 Data Quality Control

Laboratory data will be recorded on data sheets and transcribed to digital data records on the secure NOAA server within 3 business days of the date of collection and processing. This will ensure all notes are correctly updated while the events are fresh in the team's memory. Digital data files will be QC'd by a second individual alongside the data sheets to ensure all data was correctly entered within one week of data entry. Discrepancies in the data will be resolved by comparing data sheets and digital logs alongside lab notebooks that contain site and date specific information that could explain outliers in the data. A record of the lab recorder, person entering the data, and QC completion will be logged to ensure all tasks are completed prior to final data collation. Each month after QC, data will be collated with the previous data and exploratory plots updated. This will serve as an added check for potential error in the data collection and archiving process.

All laboratory data sheets will be scanned and filed on the NOAA server in the project folder. Physical copies will also be filed for archiving at the NOAA Milford Laboratory. Purely digital files (eg. sonde data, photos, etc.) will be uploaded directly to the NOAA server which is backed up regularly.

11 References

Cáceres-Martínez, J., R. Vásquez-Yeomans, G. Padilla-Lardizábal, and M. A. del Río Portilla. “Perkinsus Marinus in Pleasure Oyster Crassostrea Corteziensis from Nayarit, Pacific Coast of México.” Journal of Invertebrate Pathology 99, no. 1 (2008): 66–73. https://doi.org/10.1016/j.jip.2008.03.005.

Carnegie, R.B. and E.M. Burreson (2011) Declining impact of an introduced pathogen: *Haplosporidium nelsoni* in the oyster *Crassostrea virginica* in Chesapeake Bay. Marine Ecology Progress Series, 432: 1-15

Carnegie, R.B., 2021. Chapter 2.4.5. Infection with Perkinsus marinus, in: Manual of Diagnostic Tests for Aquatic Animals. World Organisation for Animal Health https://www.woah.org/fileadmin/Home/eng/Health_standards/aahm/current/2.4.05 P_MARINUS.pdf.

European Union Reference Laboratory. 2009. Diagnosis by histopathology of *Haplosporidium sp*. in oysters. 1st edition.

Mann, R., M. Southworth, R.B. Carnegie and R.K. Crockett. (2014) Temporal variation in fecundity and spawning in Crassostrea virginica in the Piankatank River, Virginia. Journal of Shellfisheries Research, 33: 167-176

END OF SOP

Appendix G – Histological evaluation of oyster reproductive condition SOP

1 Purpose

The purpose of this document is to provide concise guidance and standard methodology on how to evaluate oyster reproductive condition using histological techniques to determine sex and gonad development stage.

2 Scope

This SOP applies to Northeast Fisheries Science Center, Milford Laboratory staff who will be processing eastern oyster (*Crassostrea virginica*) tissue samples collected for the Long Island Sound Study (LISS) funded project to assess reproductive condition.

3 Supplies/Materials

- Gloves
- Premade histology slides
- Reporting materials

4 Equipment

- Compound microscope

5 Safety Precautions

Lab members will wear gloves to protect themselves and the histology slides being viewed. Slides will be handled with care to avoid any broken glass. If the slides are broken, lab safety procedures will be taken to ensure proper clean up and broken slides discarded in the sharps container. All lab members will be aware of the nearest first aid kit. Microscopes will remain on the bench tops.

6 Laboratory Quality Control

Multiple lab members will be analyzing the slides for reproductive conditioning at the same time to make sure the same methods are being used and results are being reported in the same manner. Slides will be analyzed in subsets over time to ensure accuracy of scoring.

7 Preparation

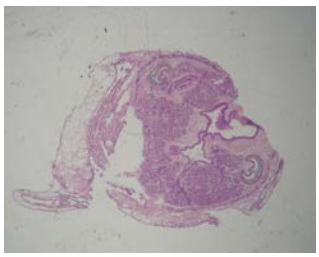
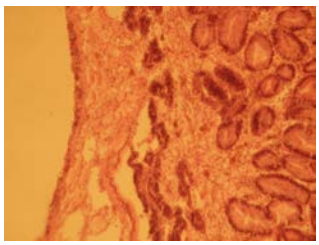
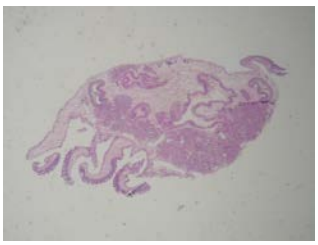
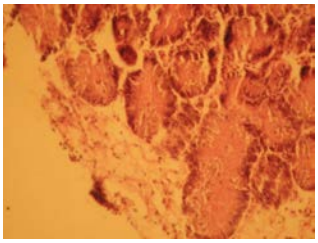
1. At least one day prior to viewing slides, examine the lab space to make sure the area is ready for the process.
2. Make sure microscopes are running properly.
3. Read through the developmental stages and review photos of each to refresh knowledge.
4. Make sure all slides have been prepped, organized, and ready to view. See See **Appendix A** and **Appendix E** for methods to prepare oyster tissues for histological preservation and slide preparation.
5. Create a list of slides/samples that will be viewed.

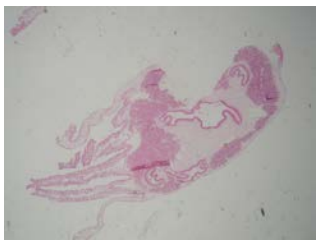
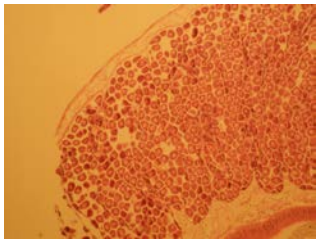
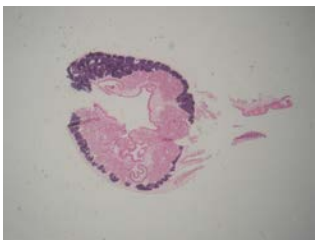
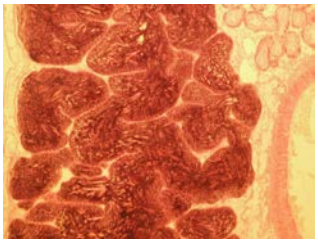
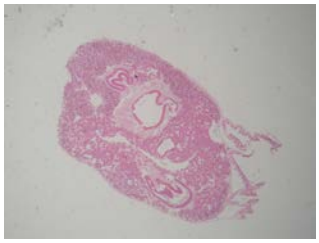
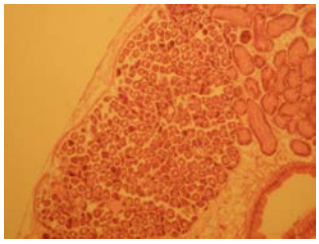
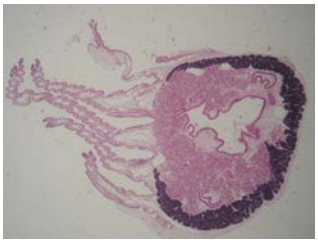
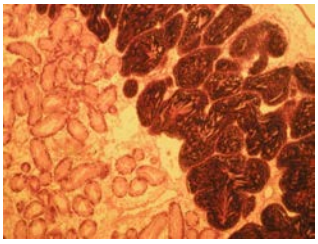
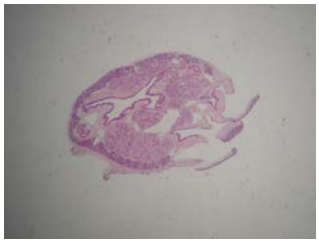
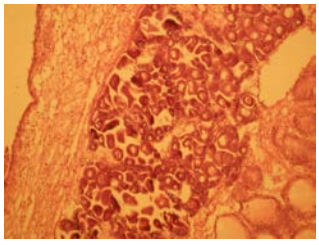
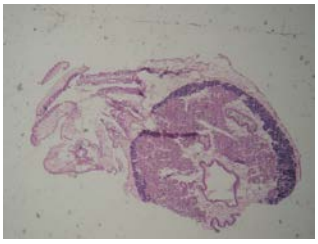
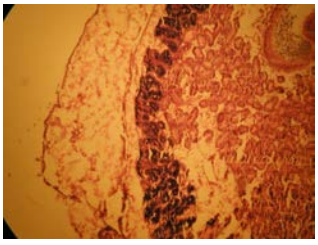
6. All data collection sheets should be organized, dated, and printed or electronically available for data recording.

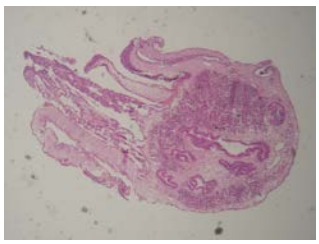
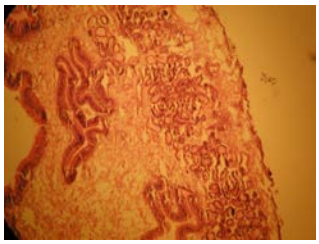
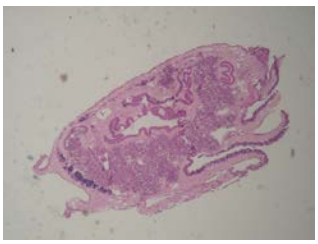
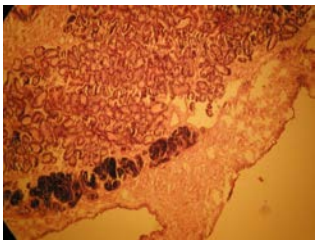
8 Procedures

1. Reproductive conditioning
    a. Using a compound microscope under 100 X total magnification, observe histology slides to determine sex and gonad development stage using methods from Gregory et al. 2023.
    b. Oyster gonad development stage is measured using a qualitative categorical scoring of gametogenesis progression. Each sample should be scored using the 5 stages listed in Table 1.

Table 1. Descriptions of gonad development stages for *Crassostrea virginica* (Gregory et al. 2023, Fisher et al. 1996; International Mussel Watch Program, 1980)

Stage number	Stage title	Description	Female	Male
0	Inactive	Follicles are nonexistent or elongated, with walls consisting of undifferentiated germinal epithelium. Sex cannot be determined		
1	Early active	Follicles contain oogonia or spermatogonia and primary oocytes or spermatocytes (no free oocytes or spermatozoa)	 	 

2	Late active	Secondary (free) oocytes and spermatocytes predominate in the follicles; there are some spermatozoa	 	 
3	Mature	Mature gametes (ova or spermatozoa) totally filling the follicles; presence of ova with distinct nucleus and nucleolus, spermatozoa oriented with tails toward the follicle lumen	 	 
4	Spawned	Follicles have gaps devoid of gametes; although numerous gametes may still remain, follicle walls may be broken. Redevelopment as evidenced by increased number of primary oocytes or spermatocytes	 	 

5	Reabsorbing	Follicles have a shrunken appearance and contain numerous phagocytes and products of reabsorption; gametes are refractory, and development is not evident	 	 

2. Results reporting
    a. The mean gonad development stage (aka Gonad Index) and sex ratios (male to female) will be calculated for each site by month. Histograms of the ratio of gonad development stages across sites and months will be produced to characterize the reproductive condition of the populations across time.
3. Clean up
    a. Wipe down all surfaces.
    b. If immersion oil was used, clean all microscope lenses and wipe down the stage.

9 Data Quality Control

Laboratory data will be recorded on data sheets and transcribed to digital data records on the secure NOAA server within 3 business days of the date of collection and processing. This will ensure all notes are correctly updated while the events are fresh in the team's memory. Digital data files will be QC'd by a second individual alongside the data sheets to ensure all data was correctly entered within one week of data entry. Discrepancies in the data will be resolved by comparing data sheets and digital logs alongside lab notebooks that contain site and date specific information that could explain outliers in the data. A record of the lab recorder, person entering the data, and QC completion will be logged to ensure all tasks are completed prior to final data collation. Each month after QC, data will be collated with the previous data and exploratory plots updated. This will serve as an added check for potential error in the data collection and archiving process.

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10 References

Gregory, K.M., McFarland, K. & Hare, M.P. Reproductive Phenology of the Eastern Oyster, Crassostrea virginica (Gmelin, 1791), Along a Temperate Estuarine Salinity Gradient. Estuaries and Coasts (2023). ttps://doi.org/10.1007/s12237-022-01163-w

Associated manuscript data and code: https://github.com/KMcFarland1/oyster

Council, N. R. The International Mussel Watch: Report of a Workshop. (1980) doi:https://doi.org/10.17226/19786.

Fisher, W. S., Winstead, J. T., Oliver, L. M., Edmiston, L., & Bailey, G. O. (1996). Physiologic Variability of Eastern Oysters from Apalachicola Bay, Florida. Journal of Shellfish Research, 15(3), 543–553.

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