



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.

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Table of Contents

Approval Signatures	1
Table of Contents	3
1. PROJECT MANAGEMENT	6
1.1 Contact Information	6
1.2 Project Objectives and Approach	9
1.2.1 Background / Project Rationale	9
1.2.2 Objectives	11
1.2.3 Approach and timeline	11
1.2.4 Study Sites	13
1.3. Data Quality Objectives	15
1.3.1 Water Quality	15
1.3.2 Oyster Bed Characteristics	16
1.3.3 Population demographics	16
1.3.4 Oyster recruitment, juvenile survival, and growth	17
1.3.5 Preparing oysters for tissue sampling	17
1.3.6 Sampling oyster tissues for pathogen detection and reproductive assays	18
1.3.7 Disease Diagnostics: Triplex qPCR assay for pathogen detection	18
1.3.8 Disease Diagnostics: Ray's Fluid Thioglycollate Medium for detection of P. marinus	18
1.3.9 Disease Diagnostics: Histology for pathogen detection and diagnosis of disease	19
1.3.10 Reproductive assessment of adult oysters	19
1.4 Quality Assurance Objective Criteria	20
1.4.1 Water-Quality	20
1.4.2 Oyster Bed Characteristics	21
1.4.3 Population demographics	22
1.4.4 Oyster recruitment, juvenile survival and growth	22
1.4.5 Disease Diagnostics	23
1.4.6 Biometrics and reproductive condition	24
1.5 Special Training/Certifications	25
1.6 Documentation and Records	26
1.6.1 Documentation and Records	26
1.6.2 Data Storage and Sharing	26
1.6.3 Permits	26
2. DATA ACQUISITION	28
2.1 Sampling Methods	28
2.1.1 Oyster bed water quality (field)	28
2.1.2 Assessment of oyster bed characteristics (field)	29



Intertidal oyster beds	29
Subtidal oyster beds	30
2.1.3 Population demographics (cover, density, mortality, and sizes) (field)	31
2.1.4 Recruitment of oyster spat (field)	33
2.1.5 Growth and survival of juvenile oysters (field)	34
2.1.6 Collection of adult oysters for disease and reproductive assessments (field)	35
2.1.7 Preparing oysters for tissue sampling (lab)	35
2.1.8 Sampling oyster tissues for pathogen detection and reproductive assays (lab)	36
2.1.9 Archive tissue for future research	37
2.2 Sampling Information	38
2.2.1 Field Documentation	38
2.2.2 Field Instrument Calibration	38
2.2.3 Decontamination Procedures	38
2.3 Sample Handling Procedures	38
2.3.1 Sample Identification	39
2.3.2 Chain of custody and sample transfer field collection to lab	40
3. ANALYTICAL REQUIREMENTS	41
3.1 Analytical Methods	41
3.1.1 Disease Diagnostics: Triplex qPCR assay for pathogen detection (lab)	41
3.1.2 Disease Diagnostics: Ray's Fluid Thioglycollate Medium assay for detection of P. marinus (lab)	41
3.1.3 Disease Diagnostics: Histology for pathogen detection and diagnosis of disease (lab)	42
3.1.4 Reproductive assessment of adult oysters (lab)	42
3.2 Laboratory Standards and Reagents	42
4. QUALITY CONTROL REQUIREMENTS	43
4.1 Measurement Performance Criteria	43
4.1.1 Field Precision	43
4.1.2 Laboratory Precision	44
4.1.3 Field Accuracy	44
4.1.4 Laboratory Accuracy	45
4.2 Internal Quality Control	45
4.3 Field Quality Control	46
4.4 Laboratory Quality Control	46
5. INSTRUMENTATION & EQUIPMENT PREVENTIVE MAINTENANCE	46
5.1 Sample Equipment Cleaning Procedures	47
5.2 Instrument and Equipment Testing Procedures and Corrective Actions	47
5.2.1 Field Equipment	47
5.2.2 Laboratory Equipment	48



6. DATA MANAGEMENT	50
6.1 Data Assessment Procedures	50
6.2 Data to be Included in QA Summary Reports	50
6.3 Reporting Format	50
7. DATA VERIFICATION AND USABILITY	52
7.1 Self-Assessment, Data System Audits	52
8. REFERENCES	53
9. APPENDICES	57
Appendix A – Water Sampling and Chemistry SOP	58
Appendix B – Oyster Bed Area, Intertidal SOP	60
Appendix C – Rugosity Measurements SOP	68
Appendix D – Oyster Bed Area, Subtidal SOP	72
Appendix E – Oyster Bed Density & Size Distribution Using Quadrats SOP	74
Appendix F – Recruitment SOP	86
Appendix G – Oyster Tissue Processing & Preservation SOP	96
Appendix H – Ray's Fluid Thioglycollate Medium (RFTM) assay for detection of Perkinsus marinus SOP	109
Appendix I – DNA Extractions SOP	120
Appendix J – Triplex qPCR assay for detection and quantification of Dermo/MSX/SSO pathogens SOP	128
Appendix K – Davidson's Fixative and Histology Slide Preparation SOP	137
Appendix L – Histological evaluation of Perkinsus marinus and Haplosporidium spp. SOP	144
Appendix M – Histological evaluation of oyster reproductive condition SOP	152
Appendix N – Monthly Sonde Maintenance SOP	157
Appendix O – Monthly HOBO Maintenance SOP	170
Appendix P – Sonde Sensor Calibration SOP	177
Appendix Q – Dissolved Inorganic Carbon Measurements (Water Chemistry)	186
Appendix R – Alkalinity Measurements (Water Chemistry)	189
Appendix S – pH Measurements (Water Chemistry)	191

1. PROJECT MANAGEMENT

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	Mariah Kachmar, AIS Inc.	mariah.kachmar@noaa.gov
QA Manager	Lisa Milke, NEFSC Milford	Lisa.milke@noaa.gov
Field Lead	Mariah Kachmar, AIS Inc.	mariah.kachmar@noaa.gov
Field/Lab Technician	Kyra Lenderman, AIS Inc.	kyra.lenderman@noaa.gov
Lab Technician	TBD, AIS Inc.	TBD
Aquaculture Technician	Isaiah Mayo, NEFSC Milford	isaiah.mayo@noaa.gov
Chemistry Technician	Genevieve Bernatchez, NEFSC Milford	genevieve.bernatchez@noaa.gov
Milford Lab Director	Gary Wikfors, NEFSC Milford	gary.wikfors@noaa.gov
Dive Coordinator	TBD	TBD
Research Chemist	Shannon Meseck, NEFSC Milford	shannon.meseck@noaa.gov
Connecticut State Shellfish Pathologist	Lydia Bienlien, Connecticut Department of Agriculture, Bureau of Aquaculture	lydia.bienlien@ct.gov
EPA Project Officer	Cayla Sullivan, EPA Region 1	Sullivan.Cayla@epa.gov

1. PROJECT MANAGEMENT

QAPP Reviewer	Elise McNally, EPA Region 1	McNally.Elise@epa.gov

PROJECT MANAGER (Mariah Kachmar) 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.

QA MANAGER (Lisa Milke) will be responsible for overseeing all data entry, data QA/QC, and final archiving of data collected throughout the duration of the project. QA manager will assume the overall responsibility for ensuring that the data meets the project objectives and quality standards outlined in the QAPP and will ensure that corrective actions are implemented, if necessary.

FIELD LEAD (Mariah Kachmar) 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. The field lead will be responsible for field and lab data management, quality control, reporting. In addition to field activities, the field lead will also play a role in managing molecular diagnostics and protocols.

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

AQUACULTURE TECHNICIAN (Isaiah Mayo) has the overall responsibility of assisting the field lead and field/lab technician with all laboratory related activities. Scope-related activities include sample collection, processing, storage, analysis, and data entry of field and laboratory collected data.

1. PROJECT MANAGEMENT

CHEMISTRY TECHNICIAN (Genevieve Bernatchez) has the overall responsibility of running discrete chemistry analysis of water samples collected monthly from each site and assisting the field lead and field/lab technician with sample collection and processing. Scope-related activities include collection and processing of samples, water chemistry analysis, and data entry.

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.

DIVE COORDINATOR (TBD) 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.

RESEARCH CHEMIST (Shannon Meseck) has the overall responsibility of assisting with the interpretation of discrete chemistry sample analysis and ensuring that all data are collected using methods in accordance with the guidelines for best practices for ocean acidification research and data reporting.

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
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1. PROJECT MANAGEMENT

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

1. PROJECT MANAGEMENT

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. 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

1. PROJECT MANAGEMENT

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, monthly full carbonate chemistry analysis, 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 continuous water quality monitoring (water temperature, depth, salinity, pH, dissolved oxygen, chlorophyll a)
- Oyster bed carbonate chemistry (monthly point samples: alkalinity, aragonite saturation state, pH, dissolved inorganic carbon)
- Oyster bed characteristics (area of coverage, 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 (spat)
- 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 2023-2-4 and 2025- 2027.

	2023-2024												2025-2027											
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

1. PROJECT MANAGEMENT

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

Task	Type	Kachmar	Milke	Lenderman	TBD
Water quality	F	C	A	R	I
Oyster bed characteristics	F	C	A	R	I
Demographics of adult oysters	F	C	A	R	I
Oyster recruitment and growth	F	C	A	R	I
Preparing adult oysters for tissue sampling	L	C	A	R	R
Tissue sampling of adult oysters	L	C	A	R	R

Molecular disease diagnostics	L	C	A	R	R
Microscopical disease diagnostics	L	C	A	R	R
Histological disease diagnostics	L	C	A	I	I
Histological reproductive assessment	L	C	A	R	R
QA/QC of field data	F	C	A	R	I
QA/QC of lab data	L	C	A	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 of the four 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

1. PROJECT MANAGEMENT

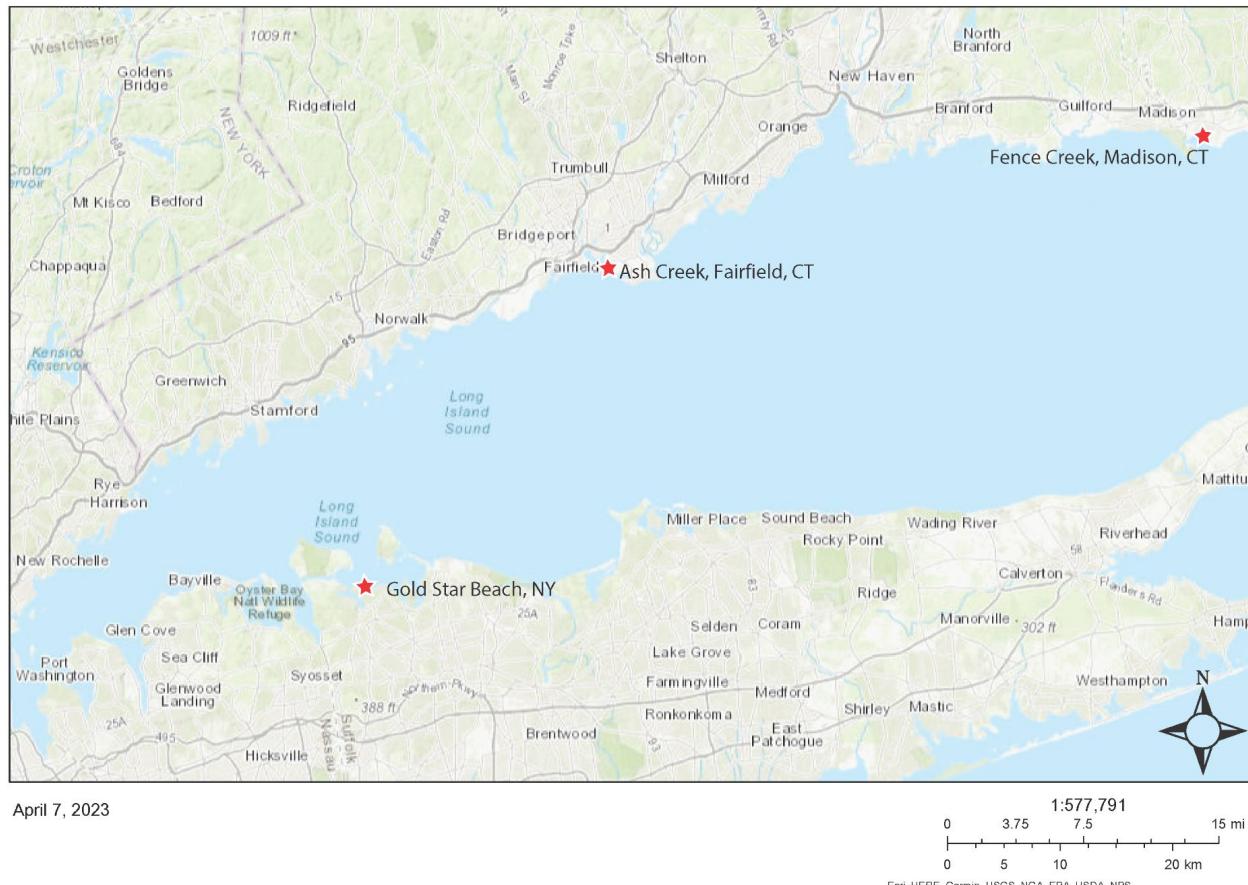
monitoring methods will be made according to the guidelines set forth in this QAPP after we are able to visually inspect the tentative sites.

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

Site Name, Town	Ash Creek, Fairfield	Fence Creek, Madison	Gold Star Battalion Beach, Huntington 2022 reef	Gold Star Battalion Beach, Huntington 2023 reef
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 Huntington, NYSDEC, NYSG
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 in September 2022	Active – planting juvenile oysters in September 2023
Recreational Harvest	Prohibited	Prohibited	Prohibited	Prohibited

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

1. PROJECT MANAGEMENT



April 7, 2023

1:577,791
0 3.75 7.5 10 20 km
Esri, HERE, Garmin, USGS, NGA, EPA, USDA, NPS

Figure 1–1. Approximate locations of finalized 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)

1. PROJECT MANAGEMENT

pH	no unit
Chlorophyll-a	Relative fluorescence units (RFU)
Dissolved Inorganic Carbon	Micromoles per kilogram ($\mu\text{mol kg}^{-1}$)
Total Alkalinity	Micromoles per kilogram ($\mu\text{mol kg}^{-1}$)
partial pressure $p\text{CO}_2$ and aragonite and calcite saturation state (Ω_{ar} and Ω_{ca} , respectively)	Micro Atmosphere (μatm)
carbon ion constituents (bicarbonate HCO_3^- and carbonate CO_3^{2-})	Micromoles per kilogram ($\mu\text{mol kg}^{-1}$)
aragonite saturation state (Ω_{ar}) and calcite saturation state (Ω_{ca})	No unit

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
Height of oyster bed (vertical relief)	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	$\frac{\text{Number of gaper oysters per meter squared}}{\text{Number of live + box + gaper oysters per meter squared}} \times 100\%$

1. PROJECT MANAGEMENT

Total mortality	$\frac{\text{Number of box + gaper oysters per meter squared}}{\text{Number of live + box + gaper oysters per meter squared}} \times 100\%$
Shell height	mm
Size composition of oysters	Percent of population per shell height category: juveniles (<40mm); young adults (40-80mm); market-size adults (80-120mm); post-market adults (>120mm).

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
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
Shell height	mm
Tissue condition	Score 1-9 (no unit)
Gross internal lesions / general observations	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. PROJECT MANAGEMENT

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)	<i>P. marinus</i>	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)	<i>H. nelsoni</i> <i>H. costale</i>	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)	<i>P. marinus</i> <i>H. nelsoni</i> <i>H. costale</i>	% (# of positive individuals / # of sampled individuals)
Weighted Prevalence (population)	<i>P. marinus</i> <i>H. nelsoni</i> <i>H. costale</i>	(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
<i>P. marinus</i> Infection intensity (individual)	Mackin Scale: 0-5 (semi-quantitative) (Ray, 1954a, 1954b, Mackin, 1962)
<i>P. marinus</i> Prevalence (population)	% (# of positive individuals / # of sampled individuals)

1. PROJECT MANAGEMENT

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

1. PROJECT MANAGEMENT

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	TidBiT MX Temp 400 HOBO	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%

1. PROJECT MANAGEMENT

pH	Aqua TROLL 600 sonde (In Situ, Inc); EPA Std. Methods 4500-H+, EPA 150.2	0.01	0.1	90%
Chlorophyll a	Aqua TROLL 600 sonde (In Situ, Inc)	0.001 RFU	0.01 RFU	90%
pH	UV-VIS spectrophotometer with m-cresol purple indicator dye	0.0001	error of ± 0.0014	90%
Dissolved Inorganic Carbon	Apollo SciTech DIC analyzer	0.01 $\mu\text{mol kg}^{-1}$	0.15% of assigned values using certified reference materials	90%
Total Alkalinity	Metrohm alkalinity titrator	0.01 $\mu\text{mol kg}^{-1}$	0.17% mean error relative to certified reference materials	90%

1.4.2 Oyster Bed Characteristics

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

Parameter	Method	Precision	Accuracy	Completeness
Latitude, longitude	Google Earth	1-4 m	RMSE <1.5%	100%
Oyster bed area, intertidal	Google Earth	1-4 m	RMSE <1.5%	100%
Oyster bed area, subtidal	Measuring tape	1 m^2	1 m^2	90%
Contoured chain length (for rugosity)	weighted chain and measuring tape	0.5 cm	0.5 cm	90%
Straight-line distance (for rugosity)	Measuring tape	0.5 cm	0.5 cm	90%

1. PROJECT MANAGEMENT

Rugosity index	<i>Contoured chain line</i>	0.01	± 0.01	90%
	<i>Straight – line distance</i>			

*RMSE = Root-Mean-Square deviation

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	Direct measurement	0.01 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	0.01 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. PROJECT MANAGEMENT

1.4.5 Disease Diagnostics

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

Parameter	Method	Sensitivity	Precision	Accuracy	Completeness
<i>P. marinus</i> prevalence	RFTM	Limit of detection: 1,000 cells per g tissue (Bushek, 1994)	<10% RPD between multiple readers	A subset of slides (10-15) per month will be scored by multiple reviewers for agreeance	90%
<i>P. marinus</i> intensity	RFTM	1 cell per sample	<10% RPD between multiple readers	Follow standard scoring criteria (modified Mackin scale); A subset of slides (10-15) per month will be scored by multiple reviewers for agreeance	90%
<i>P. marinus</i> , <i>H. nelsoni</i> , <i>H. costale</i> prevalence	qPCR (Piesz et al., 2022)	10 copies per sample	≤2.0% C _T between technical replicates	no template control	90%
<i>P. marinus</i> , <i>H. nelsoni</i> , <i>H. costale</i> intensity	qPCR (Piesz et al., 2022)	10 copies per sample	≤2.0% C _T between technical replicates	10–10 ⁸ copies standard curve	90%
(<i>H. nelsoni</i> & <i>H. costale</i>)** prevalence	Histology	Sensitivity is low for low level infections and moderate for moderate-high infections. Species	<10% RPD between multiple readers	A subset of slides (10-15) per month will be scored by multiple reviewers for agreeance	90%

1. PROJECT MANAGEMENT

		specificity is poor**			
(<i>H. nelsoni</i> & <i>H. costale</i>)** intensity	Histology	Sensitivity is low for low level infections and moderate for moderate-high infections. Species specificity is poor**	<10% RPD between multiple readers	Follow standard scoring criteria (Carnegie & Burreson 2011); A subset of slides (10-15) per month will be scored by multiple reviewers for agreeance	90%

* 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 height	Direct measurement	0.01 mm	Refer to standard	90%
Tissue condition	Visual Inspection (Quick and Mackin, 1971)	<10% RPD between multiple readers	Follow standard scoring criteria	90%
Sex	Histology	<10% RPD between multiple readers	A subset of slides (10-15) per month 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) per month will	90%

1. PROJECT MANAGEMENT

			be scored by multiple reviewers for concordance	
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* RPD = Relative Percent Difference

1.5 Special Training/Certifications

All personnel will receive training from subject matter experts and project lead Katherine McFarland prior to data collection activities. In the event that boats are required to access sites, all core team members (McFarland, Kachmar, Lenderman, Roper, Mayo, and Bernatchez) will receive NOAA vessel training, and the 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 digital tablets or 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 digital tablets and 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 transcribed from paper sheets and uploaded from the tablets to a backed up project specific and secure location on the NEFSC Google drive within 3 business days of collection. Copies of .csv, .xlsx, .hobo, and other files generated, and output from utilized software, will be uploaded to the same shared Google Drive. 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 Mariah Kachmar and Kyra Lenderman at the Milford Lab. Kyra Lenderman or Kelly Roper will be responsible for entering and securely storing all laboratory data collected on individual oyster samples (RFTM, qPCR, and disease and reproductive histology results). Mariah Kachmar will be responsible for entering and securely storing all field data (field sheets, field photographs, sonde recordings, field recordings).

1. PROJECT MANAGEMENT

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 all necessary permits to collect oysters for scientific research and will renew them as required for continued sampling. 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** - License for Scientific/Resource Assessment
License #: ACSR.0000006
Issued to: Meghana Parikh, NOAA NEFSC
Effective Date: 3/30/2023
Expiration Date: 12/31/2025
3. **New York State Department of Environmental Conservation** - License to Collect or Possess: Marine Resources
License #: 1138
Issued to: Lisa Milke, NOAA NEFSC
Effective Date: 4/1/2023
Expiration Date: 3/31/2024

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 30 minutes. Additionally, water samples will be collected monthly at each site to test specific water chemistry parameters such as pH and carbonate chemistry ([Appendix A](#)). In addition to characterizing critical ocean acidification parameters at these sites, discrete pH measurements will validate pH data collected from the Sonde and improve our ability to interpret results of continuous water quality monitoring.

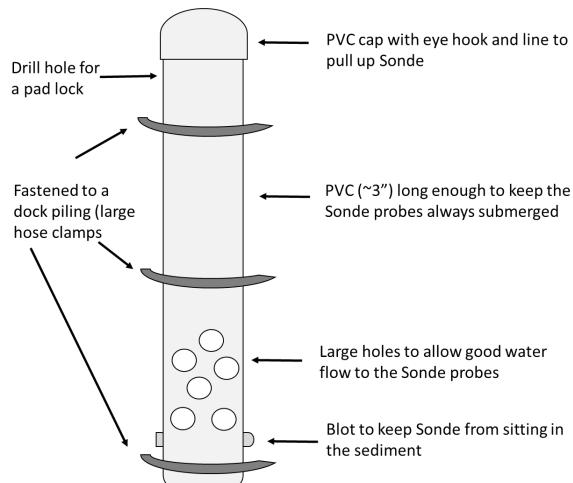


Figure 2–1. Schematic of the protective PVC housing that will be used to safely and securely deploy data sondes on derby dock pilings.

Seawater samples will be collected monthly during site visits in dark polypropylene bottles (500 mL) near the bottom where the Sonde is located to measure pH, dissolved organic carbon (DIC, $\mu\text{mol kg}^{-1}$), and total alkalinity (TA, $\mu\text{mol kg}^{-1}$) by open-cell titration (SOP 3b; Dickson et al., 2007). DIC will be measured on an Apollo SciTech DIC analyzer Model AS-C5 (Apollo SciTech,

2. DATA ACQUISITION

LLC, Newark, DE) with precision of 0.5% of assigned values in an interlaboratory comparison (Bockmon & Dickson, 2015) ([Appendix Q](#)). TA will be measured using certified HCl titrant ($\square 0.1 \text{ mol kg}^{-1}$, $\square 0.6 \text{ mol kg}^{-1}$ NaCl; Dickson Lab, Batches 191 and 157) on a Metrohm alkalinity titrator (Mettler Toledo T5) with 0.17% mean error relative to certified reference materials (Dickson Lab CO₂ CRM Batch 191 and 157) ([Appendix R](#)). A UV-VIS spectrophotometer (Cary100, Agilent, Santa Clara, CA, USA) will be used to determine pH colorimetrically at 20°C with m-cresol purple indicator dye (Sigma-Aldrich, St. Louise, MS, Dickson & Goyet, 1994), with an error of ± 0.0014 (seawater scale) ([Appendix S](#)). Seawater DIC and pH were used in CO2SYS (Pierrot et al., 2006) for the calculation of partial pressure $p\text{CO}_2$ (μatm), carbon ion constituents (bicarbonate HCO_3^- and carbonate CO_3^{2-} , $\mu\text{mol kg}^{-1}$), and aragonite and calcite saturation state (Ω_{ar} and Ω_{ca} , respectively) using the following constants: K1, K2 from Lueker et al. (2000); potassium sulfate from Dickson (1990); and boron from Lee et al. (2010).

2.1.2 Assessment of oyster bed characteristics (field)

Sampling sites will be evaluated for total area of oyster bed coverage and oyster bed height above surrounding substrate. 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 annually in May-June.

Intertidal oyster beds

To determine the area of oyster bed coverage, handheld GPS will be used to estimate the area of the oyster bed by walking the perimeter of the bed (defined as the continuous edge where live or dead shell make up ~25% of the substrate; Janiak, 2021). Area of the oyster bed will be calculated from the resulting polygon. See [Appendix B](#) for detailed SOP.

To quantify oyster bed rugosity, measurements will be taken from a minimum of three 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 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:

$$\text{Rugosity (intertidal)} = \frac{\text{Measured contoured chain length}}{\text{Fixed chain length}}$$

The rugosity index will be between 0 and 1, where oyster beds with values closer to 1 demonstrate less complex surfaces (the contoured chain distance is closer to the fixed length). Values closer to 0 indicate a more complex surface (contoured chain covers a shorter distance). See [Appendix C](#) for detailed SOP.

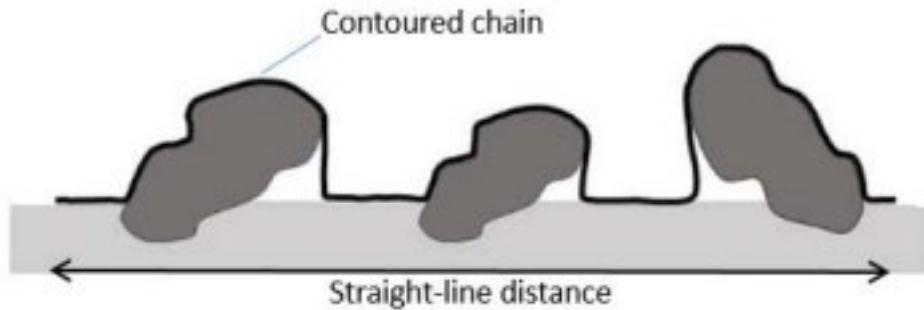


Figure 2–2. Schematic showing method of measuring oyster bed rugosity. A fine-link chain will be conformed to the surface of the oyster bed without pushing the chain into overhang crevices. Rugosity will be calculated as the measured contoured chain length divided by the fixed chain length (Janiak, 2021).

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. See [Appendix D](#) for detailed SOP.

Rugosity (surface complexity) will be quantified similarly to the method described for intertidal oyster beds. A fine link chain will be attached to a weighted quadrat to assist divers in maintaining control in the current. The chain will be laid diagonally across the quadrat to conform to the surface of the oyster bed. The point where the chain reaches the other end of the quadrat will be marked with a zip-tie (**Figure 2–3**). At the surface, this distance between the chain attachment and the ziptie will be measured (measured diagonal length). The fixed diagonal length is a known distance and both measurements will be used to calculate rugosity as follows.

$$\text{Rugosity (subtidal)} = \frac{\text{Fixed diagonal length}}{\text{Measured diagonal length}}$$

2. DATA ACQUISITION

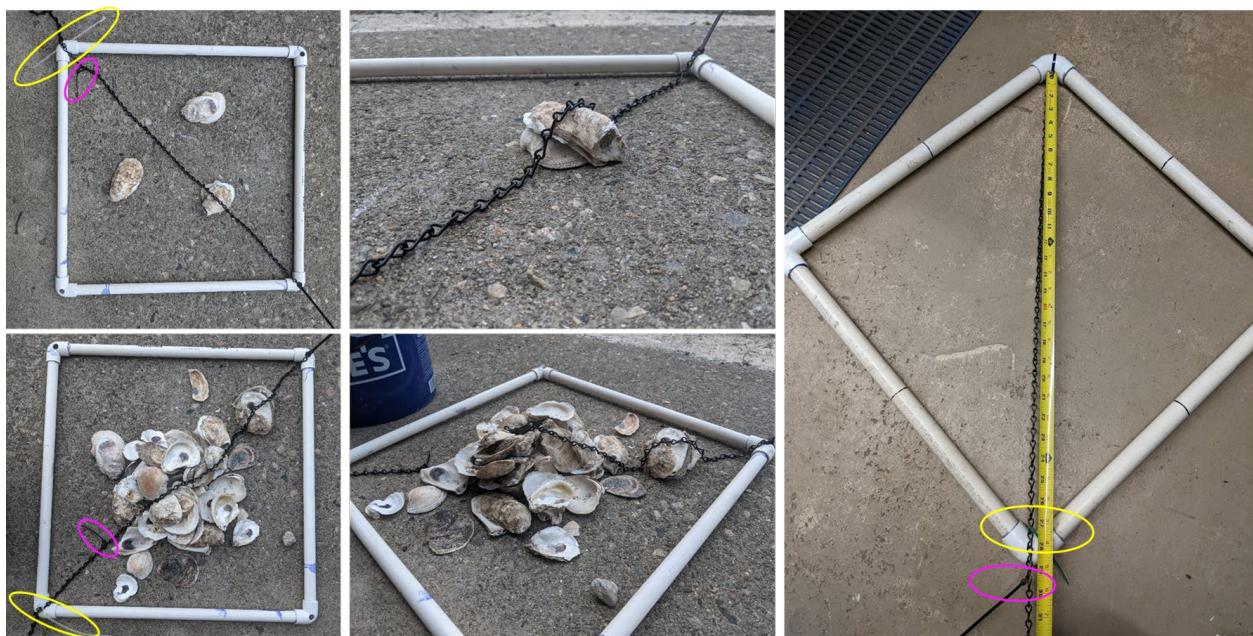


Figure 2–3. Example of rugosity measurements on low profile and small mound oyster beds. Pink circles mark the length of the chain taught along the diagonal of the quadrat (fixed diagonal length, marked by a black zip tie) and yellow circles mark added zip ties (measured diagonal length). Divers will mark the chain, and the distance between the attachment point and the added zip ties will be measured by the shore team.

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 field seasons. This will allow demographic parameters to be determined before and after the expected peak onset of MSX disease (July-August) (Burreson & Stokes, 2006) and Dermo 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 (<10 mm), and all juveniles (shell height ≥ 10 mm and < 40 mm). 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 mortality.

Samples will be taken from randomly placed quadrats. A minimum of three quadrat samples will be taken per site and sampling event using a 25 x 25 cm weighted PVC quadrat. Once a quadrat has been placed, the percent of area covered by articulated oysters (live, gapers, and box combined) will be visually estimated and recorded prior to excavation. Articulated oysters will then be excavated from quadrats and enumerated by oyster category (**Table 2–1**). Up to 100 individuals across all categories will be measured using calipers to the nearest 0.1 millimeter for shell height as shown in **Figure 2–4**. Mortality indices (adapted from Ford et al., 2006) will be calculated as follows:

2. DATA ACQUISITION

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

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

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). See [Appendix E](#) for detailed SOP.

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.

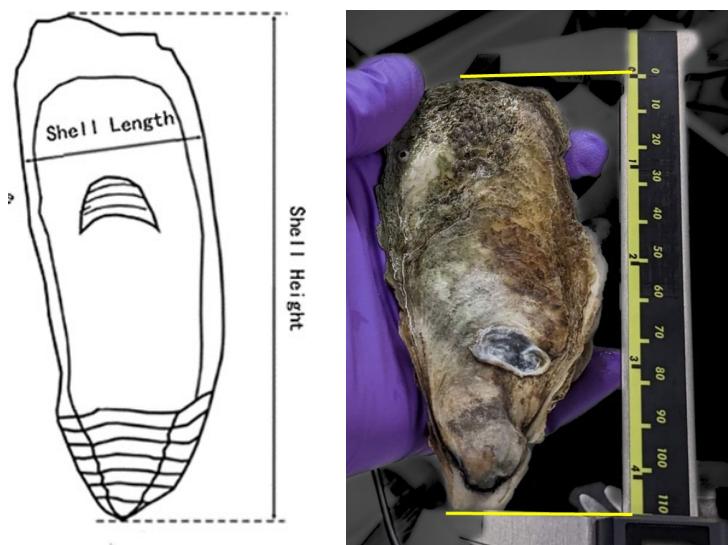


Figure 2–4. Oyster shell height will be measured in the field and for lab specimens.

2. DATA ACQUISITION

2.1.4 Recruitment of oyster spat (field)

Oyster recruitment will be quantified at each site using oyster shell stringers (**Figure 2–5**). Each shell stringer will consist of 6 cured oyster shells (shell heights, 100–120mm) 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). Cured shells will be sourced from the Ash Creek Shell Recycling program and will have a minimum of 6 months curing duration on land to ensure no spread of disease of invasive species with the shells.

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 six shell strings (6 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 stringers; October for the August-deployed stringers). At the time when recruitment is assessed, shell stringers will be returned to the laboratory so that oyster spat can be verified under a magnifier light. Recruitment will be quantified as the mean number of newly settled oysters per oyster shell, deployment date, and site. See [Appendix F](#) for detailed SOP.



Figure 2–5. Example of shell stringer attached to T-bar. (Photo from Florida Fish and Wildlife Conservation Commission)

2.1.5 Growth and survival of juvenile oysters (field)

During peak oyster spat settlement (June–October), 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 mm² openings) filled with whole, unarticulated cured shells to serve as a substrate for spat settlement (McFarland and Hare, 2018; **Figure 2–6**).

Annually in October, 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. In spring, spat shell heights will be measured to the nearest 0.01 mm using digital calipers. Spat will be

2. DATA ACQUISITION

distributed equally among three replicate bags, with the number of shells being dependent on spat density (goal of 50–100 spat per bag). Bags will be secured to the seabed using metal stakes/rebar adjacent to the oyster bed where the spat were collected. Spat will not be moved among sites.

From April-October, regular counts of the bagged juvenile oysters (those that settled during the previous summer/fall season) 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 during each survival count (with replacement, as above) to monitor mean growth rates. Any new spat that settle inside the cages (identified as those with a shell height less than the previous month's minimum) will be removed from the shell 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.



Figure 2-6. Shell bags used by McFarland and Hare (2018) to collect spat for growth experiments. Shell bags were made of polypropylene mesh (~15 mm² 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 at subtidal sites in NY and year round at intertidal sites in CT (2024-2027) 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. Up to 3 oysters will be kept per quadrat and oysters not kept from

2. DATA ACQUISITION

quadrat samples will be returned to the oyster bed. 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 G](#). Upon arrival at the Milford Laboratory, oysters will be processed immediately, or refrigerated at 4°C 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. For each individual shell height will be recorded to the nearest 0.1 millimeter (**Figure 2–4**). Once shucked, oysters will be assigned a body condition scoring of 1 through 9 to indicate overall health of the individual (Quick and Mackin 1971, [Appendix G](#)).

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 (**Figure 2–7**). 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 5×5 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 G](#)).

2. DATA ACQUISITION

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)	<i>P. marinus</i> <i>P. nelsoni</i> <i>H. costale</i>	Gill, gut, and rectal tissue	0.5 mg (combined)	Frozen at -80°C or 95% ethanol
Microscopy	<i>P. marinus</i>	Mantle adjacent to the palps	5x5 mm section	Ray's fluid thioglycollate medium (RFTM), 5-7 days
Histology	<i>P. marinus</i> <i>P. nelsoni</i> <i>H. costale</i> 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

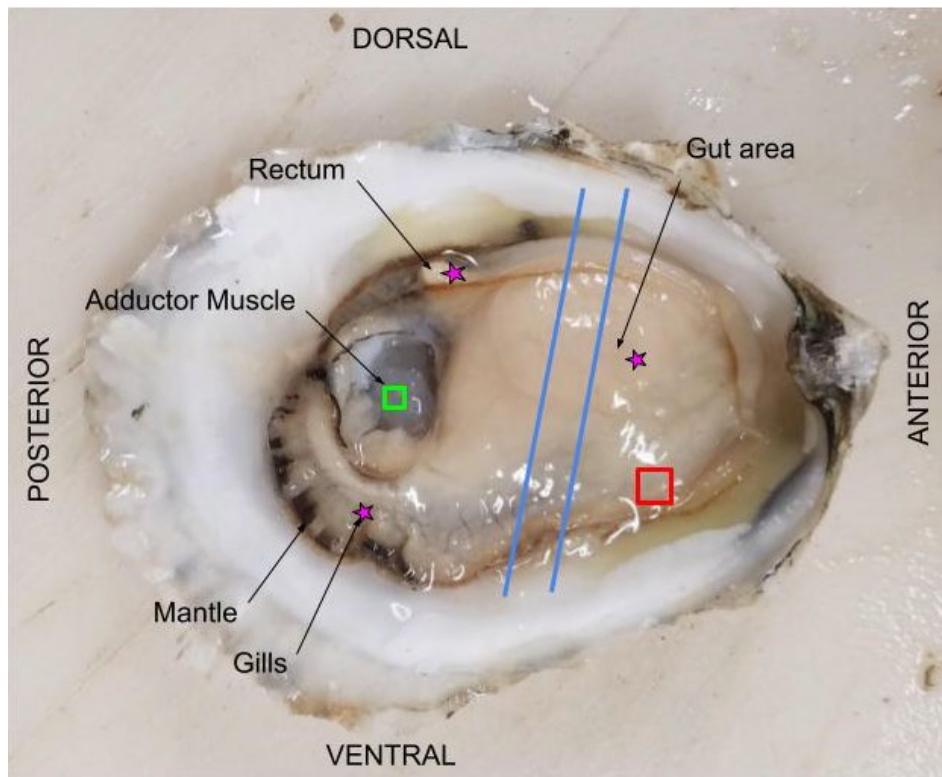


Figure 2–7. *Crassostrea virginica* anatomy showing tissues that will be sampled for disease diagnostics and reproductive conditioning. Tissue between the blue parallel lines will be sampled for histology (includes mantle, digestive tract, gonads, kidney, and gills). The red square shows mantle tissue 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 mm³) will be dissected and preserved in 95% Ethanol (see [Appendix G](#) 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 of this document.

2.2.1 Field Documentation

All field data will be documented on a digital tablet or in a waterproof field notebook. Field notebooks 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. DATA ACQUISITION

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 G](#)). 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 G](#)) 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. Hold fresh frozen tissue up to 1 year at -80°C.
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°C (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°C.
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. DATA ACQUISITION

2.3.1 Sample Identification

All tissue samples will be labeled with the following information:

1. Collection site
2. Collection month and year (MM/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 donated to the local CT shell recycling program.

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 (**Appendices H–M**) 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 I & J**). 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**).

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

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

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

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 H**). Population level metrics of infection prevalence and intensity will be derived as described in **Section 3.1.1**.

3. ANALYTICAL REQUIREMENTS

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 K](#)). Slides will be examined microscopically for presence of *P. marinus*, *H. nelsoni*, and *H. costale*, as well as tissue changes indicative of infection ([Appendix L](#)). *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 M](#). 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 0.25 x 0.25 m quadrats (e.g., McFarland et al., 2022) in random locations across the oyster bed 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:

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

where 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 shell heights of oysters (**Figure 2-3**) collected during quadrat counts will be measured to the nearest 0.01 millimeter using digital 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.

4. QUALITY CONTROL REQUIREMENTS

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 $\geq 10\%$ 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 ensure precision of the sample results. Technical replicates should have $\leq 2.0\%$ 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 and cleaning 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 Manager 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 $\geq 10\%$ between reader

4. QUALITY CONTROL REQUIREMENTS

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. PI 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 10–10⁸ 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 any data collected in the field (notebooks and data sheets) and ensuring successful upload of digital data collection 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

4. QUALITY CONTROL REQUIREMENTS

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 G](#)) in a designated area of the Biosafety Level 2 lab space. 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 PREVENTATIVE MAINTENANCE

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 (see **Appendices N & O** for detailed maintenance protocols). 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

See [Appendix P](#) for detailed descriptions for all sensor calibrations

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. During deployment, pH will be calibrated 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. 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 ([Appendix P](#)).

In-Situ provides several methods for calibrating their DO sensor. We will use the water saturated air (damp sponge) method 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) and replace as needed (expected life of 24 months according to the In-Situ [specification sheet](#)). When replacements are necessary, 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 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

5. INSTRUMENTATION & EQUIPMENT PREVENTATIVE MAINTENANCE

technical support team (pers. comm., 4/7/2023; [Appendix O](#)), and by the In-Situ technical support team (per comm., 4/10/2023).

During winter months (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 30 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 the Maintenance and Troubleshooting of the [CFX Opus Operation Manual](#) (pg. 141).

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.

5. INSTRUMENTATION & EQUIPMENT PREVENTATIVE MAINTENANCE

The Apollo SciTech DIC analyzer Model AS-C5 will be maintained and calibrated according to manufacturer recommendations. For each round of analysis, a certified reference material (Dickson Lab CO₂ CRM Batch 191 and 157) will be run in three concentrations to create a calibration curve.

The Metrohm alkalinity titrator (Mettler Toledo T5) will be maintained and calibrated according to manufacturer recommendations. Daily calibrations will be completed prior to running samples using certified reference materials in multiple dilutions to create a calibration curve (Dickson Lab CO₂ CRM Batch 191 and 157).

The UV-VIS spectrophotometer will be maintained and calibrated according to manufacturer recommendations.

Temperatures of 4°C refrigerators as well as -20°C and -80°C freezers will be regularly monitored, cleaned, 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. These 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

6. DATA MANAGEMENT

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, chlorophyll a, and carbonate chemistry 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.
- Burreson, E.M., Stokes, N.A. (2006) 5.2.2 Haplosporidiosis of oysters, In: Executive Committee (ed.) Fish Health Section Blue Book, 2014 Edition, Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens. Section 1, Diagnostic Procedures for Finfish and Shellfish Pathogens. Chapter 5 Diseases of Molluscan Shellfish. American Fisheries Society's Fish Health Section.
- 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](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.

9. APPENDICES

- 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:

9. APPENDICES

- 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>
- Quick, J. A., and J. G. Mackin (1971) Oyster parasitism by *Labyrinthomyxa marina* in Florida. Fl. Dept. Nat. Resour. Mar. Res. Lab. Prof. Pap. Ser., 13:1-55.
- 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.

9. APPENDICES

- 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 – Water Sampling and Chemistry SOP

1 Purpose

The purpose of this SOP is to provide concise guidance and methodology for collecting water samples at the oyster bed sites for water quality testing.

2 Scope

This SOP is pertaining to the EPA Long Island Sound Study funded Oyster Health project where water quality metrics will be incorporated to understand host-pathogen-environment relationships.

3 Definitions/Acronyms

4 Safety Precautions

All survey team members will wear appropriate clothing dependent on weather conditions including but not limited to waders, rubber boots or protective footwear, gloves, hats, sunglasses, long sleeve shirts, and pants. Thick protective gloves (e.g garden gloves) should be worn when handling oysters and other fouling organisms. Team members will wash hands thoroughly after field trips end. A first aid kit will be present for any injury. Extra water will be provided to avoid dehydration or heat stroke. Team members will take regular breaks when needed.

Exercise weather-appropriate field safety measures by monitoring conditions before and during the trip. Do not perform fieldwork during dangerous conditions (e.g. lightning, extreme winds, extreme floods). Do not visit field sites alone (use buddy system). Inform PIs of dates and times of fieldwork. Confirm safe return to the lab. At intertidal sites, perform procedures during low tide. At subtidal sites, divers are to follow NOAA diving regulations according to the instructions of the lab diving coordinator (Barry Smith).

5 Supplies/Materials

- 250mL amber nalgene bottle
- cooler with ice
- data sonde
- 60mL (60cc) syringes
- 0.45 μ m syringe tip filter

6 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, field notebooks will include a checklist of all data that needs to be recorded during each visit. All datasheets will be screenshotted as back up in the event data is lost before connecting to the network.

7 Procedures

1. In Field (<24 hours storage before laboratory processing):

- a. Take the 250 mL nalgene amber bottle with the appropriate site label (Figure 1) out to the sonde station
 - i. Prelabeled and cleaned amber bottles are kept in the oyster health study field gear station in the garage on the bottle shelf.

APPENDIX A

- b. Fill and rinse the bottle 3 times with surrounding sea water
 - c. Reaching to the approximate depth of where the sonde records measurements, fill the bottle to the very top and cap under water
 - d. Store the bottle in the cooler on ice for transport back to the lab
 - e. Record salinity and temperature at the site with a handheld YSI or last 5 reads of the downloaded sonde data (See Sonde SOP).
2. In Field (>24 hours storage before laboratory processing):
 - a. Fill 5 60mL (60 cc) syringes from the desired location (near Sonde deployment) and cap them under water.
 - b. On shore, attach a 0.45 µm filter to the end of each syringe and filter them into a clean, prelabeled 250 mL amber nalgene bottle.
 - c. Store the bottle in the cooler on ice for transport back to the lab
 - d. Record salinity and temperature at the site with a handheld YSI or last 5 reads of the downloaded sonde data (See Sonde SOP).
 3. Laboratory:
 - a. *Storage: Upon returning to the lab, move the bottles immediately to the fridge next to the fume hood in Building 2 Room 20 (second floor) and alert Genevieve (tex/call 617-780-7289 or email genevieve.bernatchez@noaa.gov).*
 - b. *Samples should ideally be processed within 24 hrs of collection, but if refrigerated and filtered can be processed up to 48 hrs after collection without chemically treating the sample.*



Figure 1: Water chemistry sampling bottles.

END OF SOP

Appendix B – Oyster Bed Area, Intertidal SOP

1. Purpose

The purpose of this SOP is to provide concise guidance and methodology on how to measure oyster bed area for intertidal sites using a handheld GPS and Google Earth.

2. Scope

This SOP is pertaining to the EPA Long Island Sound Study funded Oyster Health project where oyster bed area will be used to characterize the size of the oyster bed each oyster site as part of biannual population surveys.

3. Definitions/Acronyms

4. Safety Precautions

N/A

5. Supplies/Equipment

- Laptop or tablet
- Access to Google Earth online
- Handheld GPS (Garmin eTrex® 32x Rugged Handheld GPS with Compass and Barometric Altimeter + glass covers, cat # 010-01199-10)

6. 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. All datasheets will be screenshotted as back up in the event data are lost before connecting to the network.

7. Procedures

1. GPS calculations

- a. Turn the GPS on by pressing the power button on the right side (Figure 1. this button also adjusts the brightness of the screen).



Figure 1: Image of GPS showing the location of the power button and the home screen.

- b. Once the GPS is turned on, scroll down using the toggle until you find “Area Calculation”. Click on the application and press start (Figure 2).

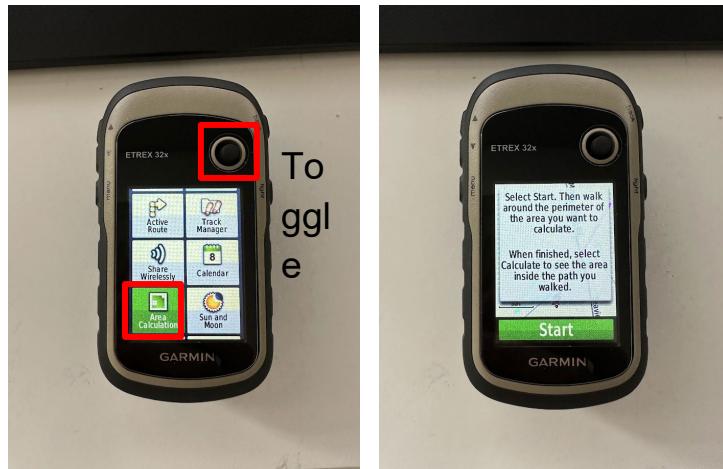


Figure 2: Image of the GPS showing the location of the Toggle to navigate to the Area Calculator tool (Left). Screen once Area Calculator tool is selected (Right)

- c. Once you have pressed start, walk the perimeter of the reef until you have reached your initial starting point again. Press “Calculate”.
- d. This GPS will calculate the area. Change the metrics to square meters. Write this number down in the field notebook.
- e. Save the file by pressing “Save Track” (Figure 3).
 - i. This will allow you to change the file name. Once the name is changed press “Done”. This file will save as a GPX file.
 1. For Ash Creek - MMYYSite_Right or _Left
 2. Other sites - MMYYSite

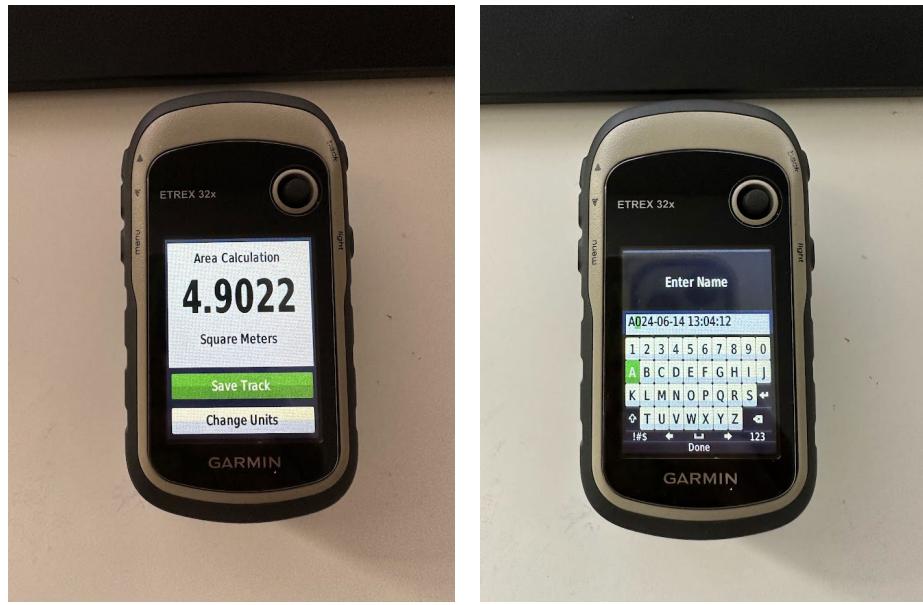


Figure 3: Image showing the results of the area calculator and how to save the Track file on the GPS.

- f. To view the track, use the menu button to return to the main screen. Scroll up to "Track Manager". This is where you will see a list of all saved tracks.
2. Downloading GPS track files to computer
 - a. Connect the GPS to a laptop by using a USB Mini cord, plugged into the port on the back of the GPS (Figure 4) and the laptop's USB port.



Figure 4: Image depicting the location of the USB mini cord port on the back of the GPS.

- b. Once the GPS is plugged in, open File Explorer application on your laptop.

- c. In the Navigation Pane located on the left side of window, there is a file labeled “Garmin eTrex 32x (D:)” towards the bottom of the pane. Click on that file.
 - i. Note: the file may take a couple minutes to appear.
- d. Click on the file labeled “Garmin”, then “GPX”. You will then see the named track files.
- e. Right click and copy the file you would like to download and paste it into Downloads.
- f. This file will be downloaded as a GPX and needs to be converted to a KML for easier exporting. Go to <https://mygeodata.cloud/converter/> and click on “or browse files to convert”, then “Add files”. Double-click on the file you want to convert and click “Continue”.
- g. On the next page click “Convert now!”
 - i. Note: The “1. Input Data” and “2. Output Data” do not need to be changed.
- h. Once converted, you will be redirected to the “Conversion Results” page. Then click “Download” to have the newly converted KML file downloaded on your computer.
- i. Once the file has been converted and downloaded to your computer, it will appear as a zip file. Open the zip file to find the KML file.
- j. Go into the Bed Area folder within the google drive and upload the new KML file. It should be named “MMYYSite”.

3. Google Earth - Creating a new project

- a. Open Google Earth (online version: earth.google.com).
- b. In the left toolbar, create a new project. Click “+ New” (Figure 5).
- c. Once the project has been created, update the project description to reflect the purpose and goals. Click the pencil icon to edit project details (Figure 6).

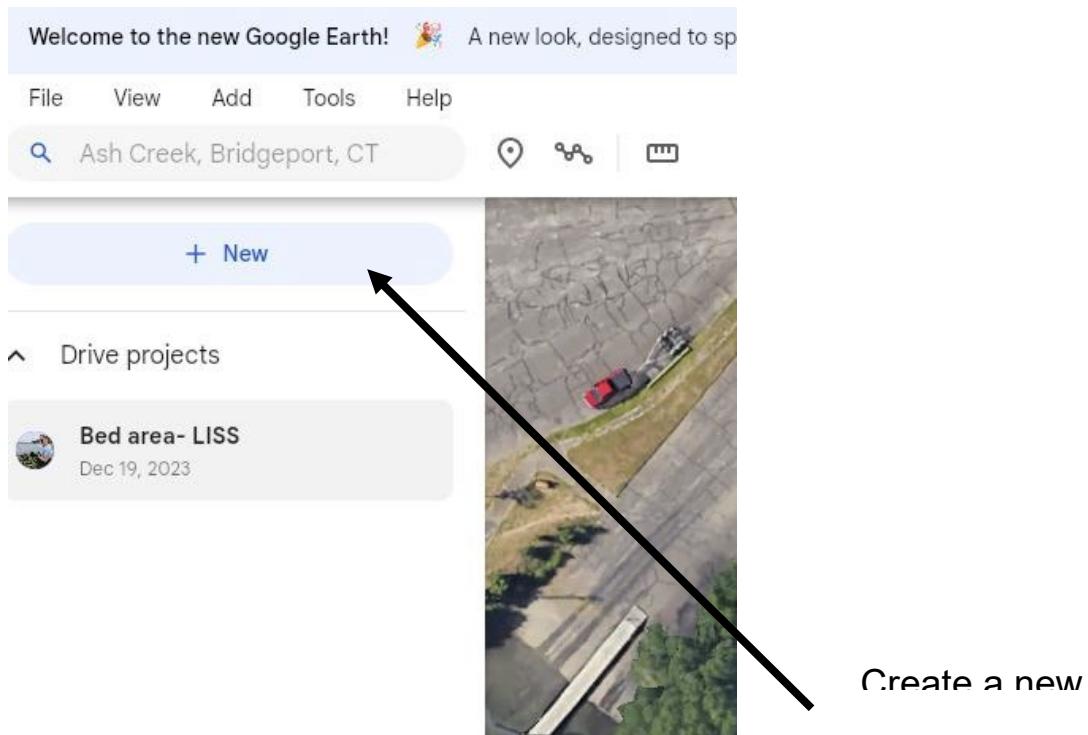


Figure 5: Depiction of where to create a new project in Google Earth.

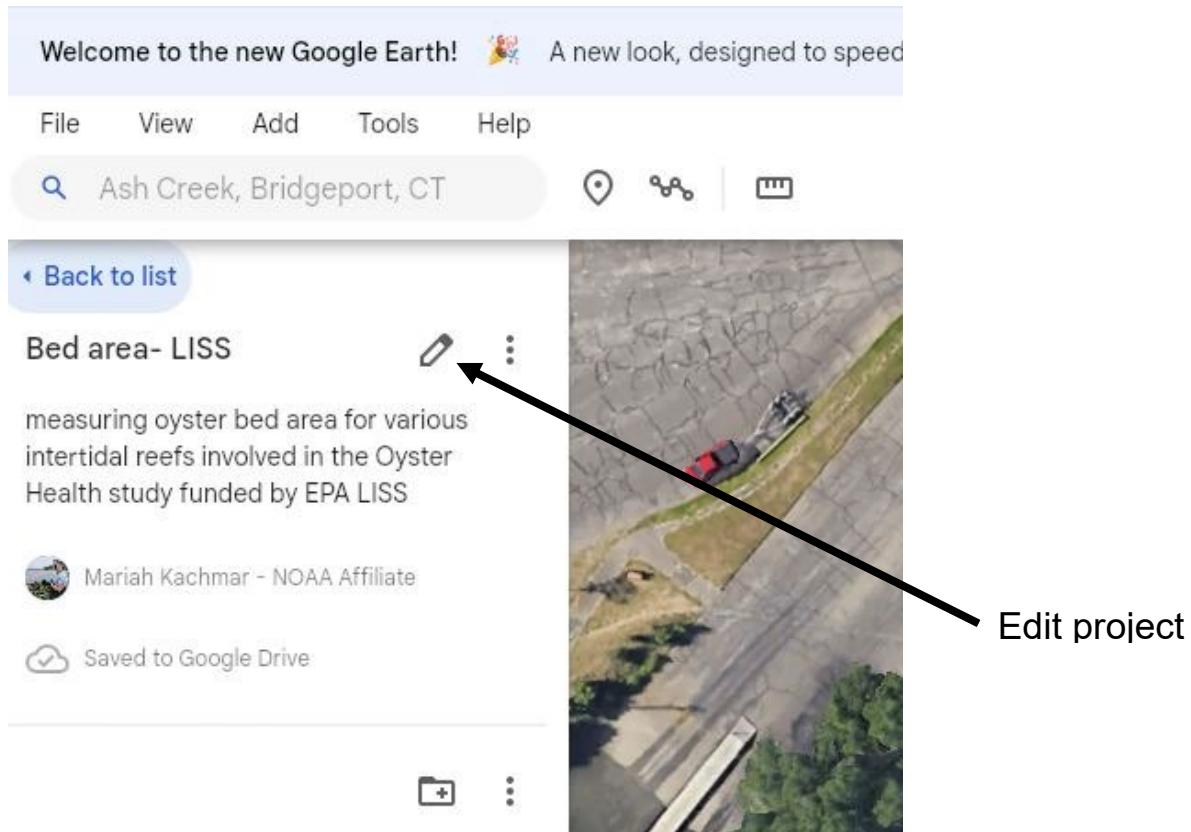


Figure 6: Depiction of where to locate the 'Edit Project' tool to edit the description.

4. Uploading GPS coordinates to Google Earth

- Next, go to Google Earth Bed Area project, and upload the KML file to google earth. Go to ‘File’ and ‘Import KML/KMZ file’ (Figure 7).

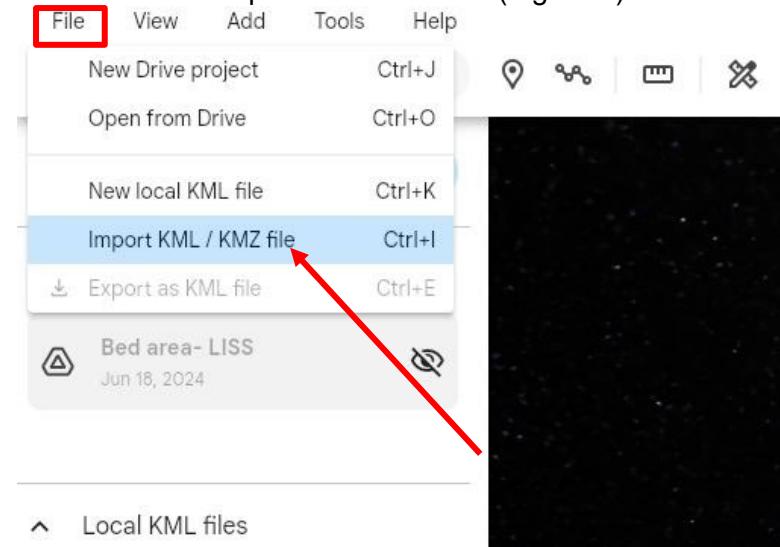


Figure 7: Depiction of where to find the “Import KML” tool.

- Once the file has been uploaded, click the file and it will take you to the site. Coordinate points should appear as yellow pins and a red line of a polygon (Figure 8).

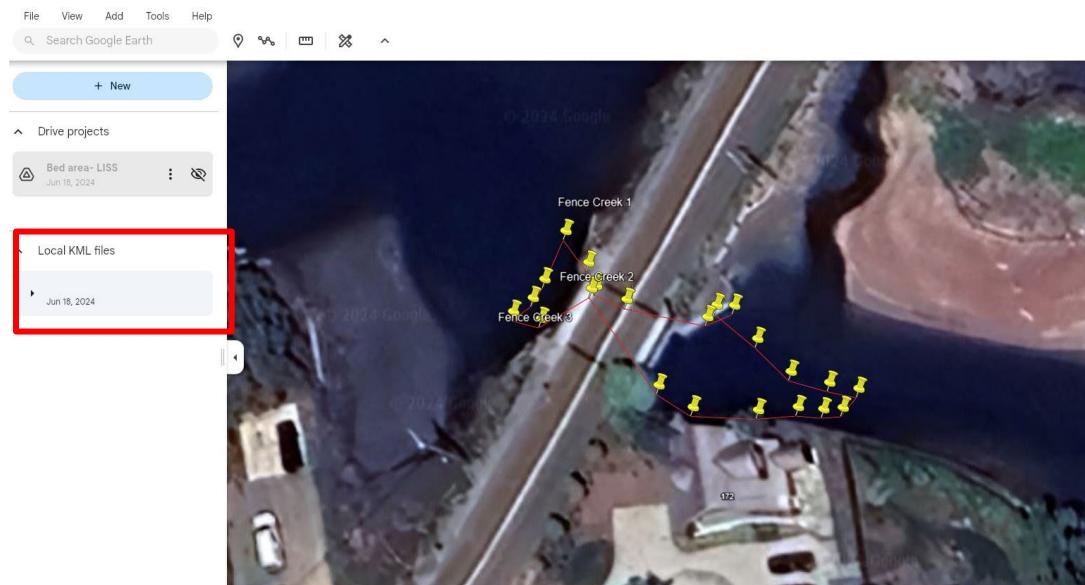


Figure 8: Depiction on where to find the KML file in Google Earth to select once it has been imported.

- Zoom in as needed. Use the zoom icons (+ or - in bottom right corner) and mouse to drag and focus the view onto the desired reef area.

5. Measuring the area of the oyster bed

- a. Once the reef has been aerially identified, click the ruler icon or polygon tool to measure distance and area (Figure 9).

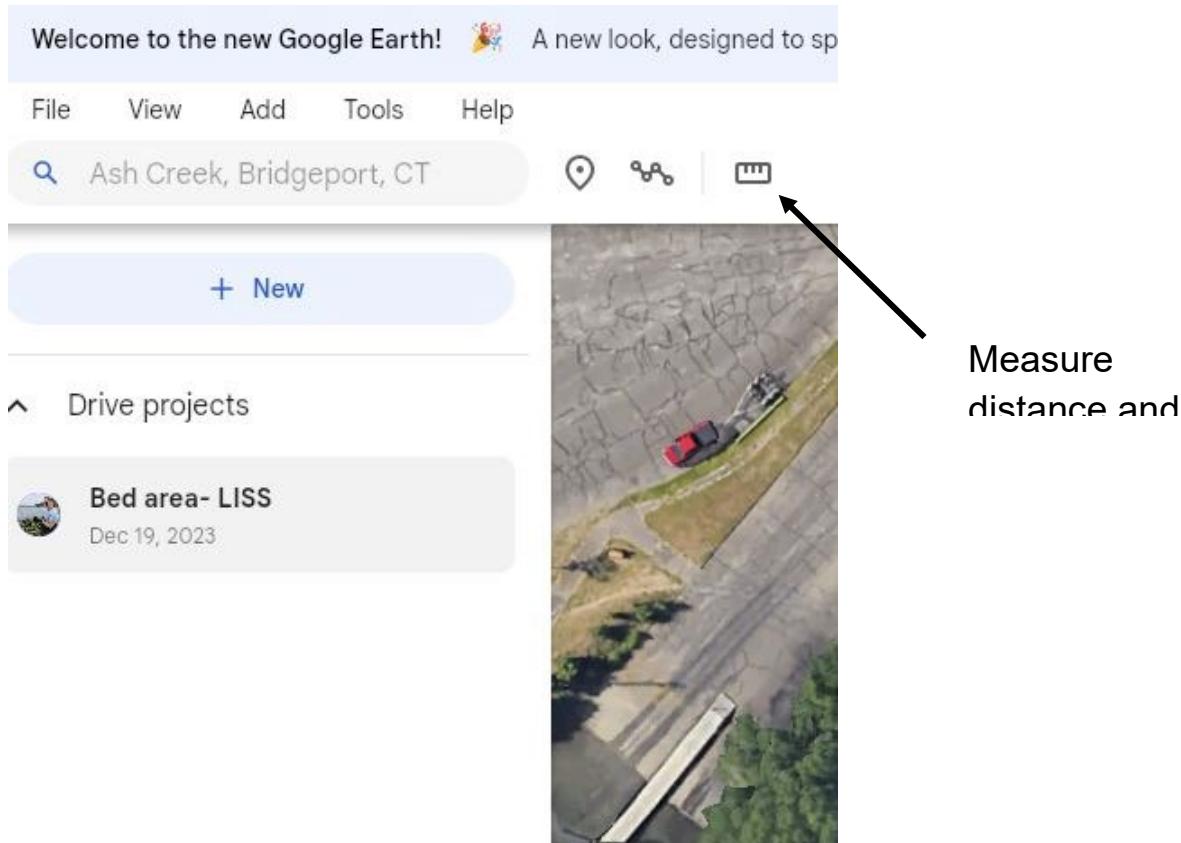


Figure 9: Depiction of where to find the 'Measure distance and area' tool to measure the area of
the oyster bed.

- b. Using the cursor, click to contour/trace the red lines using the yellow pins as the connection points. Connect all lines to create a polygon like shape (Figure 10).

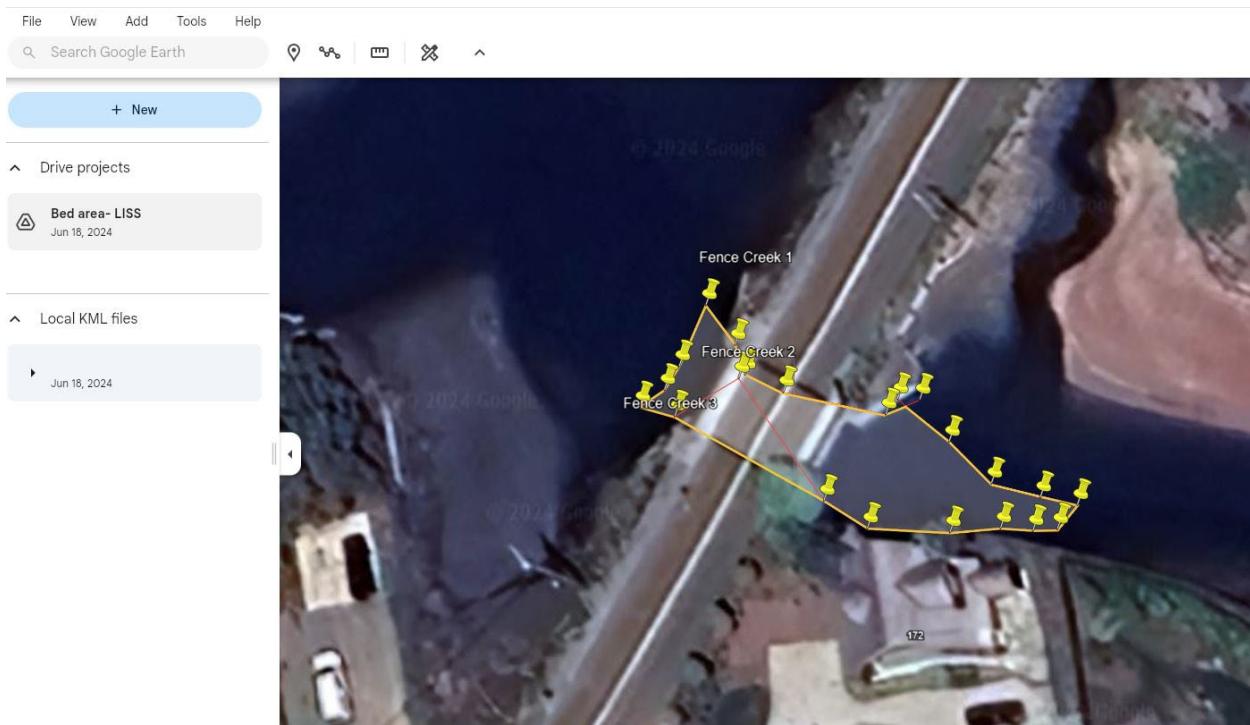


Figure 10: Depiction of the polygon formed using the 'Measure distance and area' tool.

- c. Once this shape has been formed, Google Earth will generate a perimeter measurement in meters and an area measurement in meters squared. Save this measurement to the project. The measurement should be saved as MMYYSite (Figure 11).

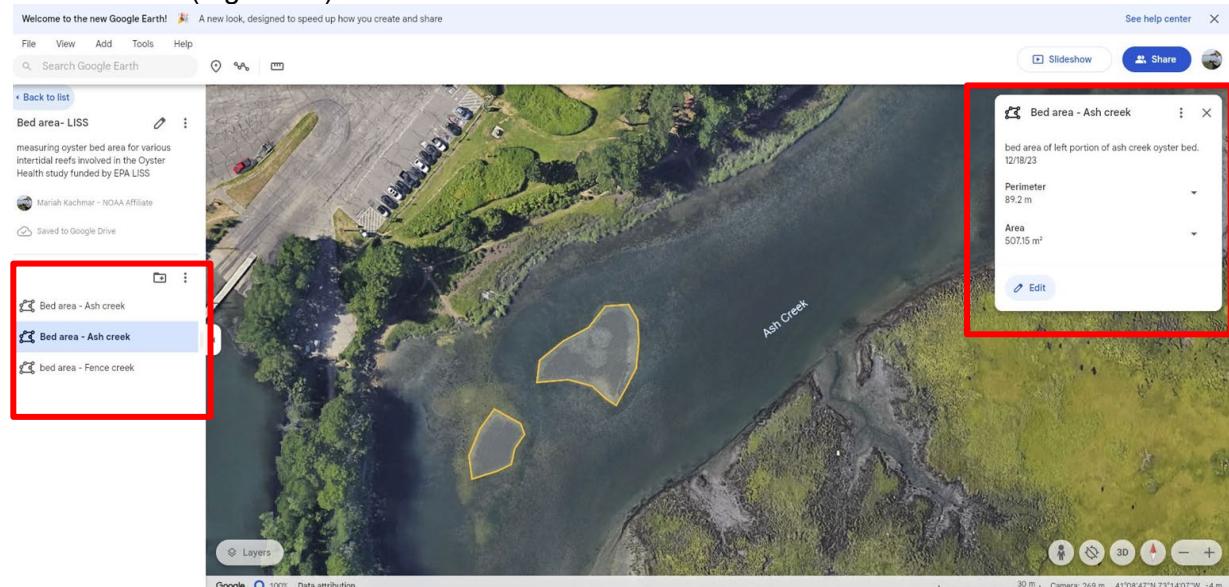


Figure 11: Depiction of a saved measurement and the data generated by Google Earth.

- d. Once the measurements have been saved, click the edit button to edit the measurement and add detailed descriptions of when the measurement was taken and what oyster reef this measurement pertains to (Figure 12).

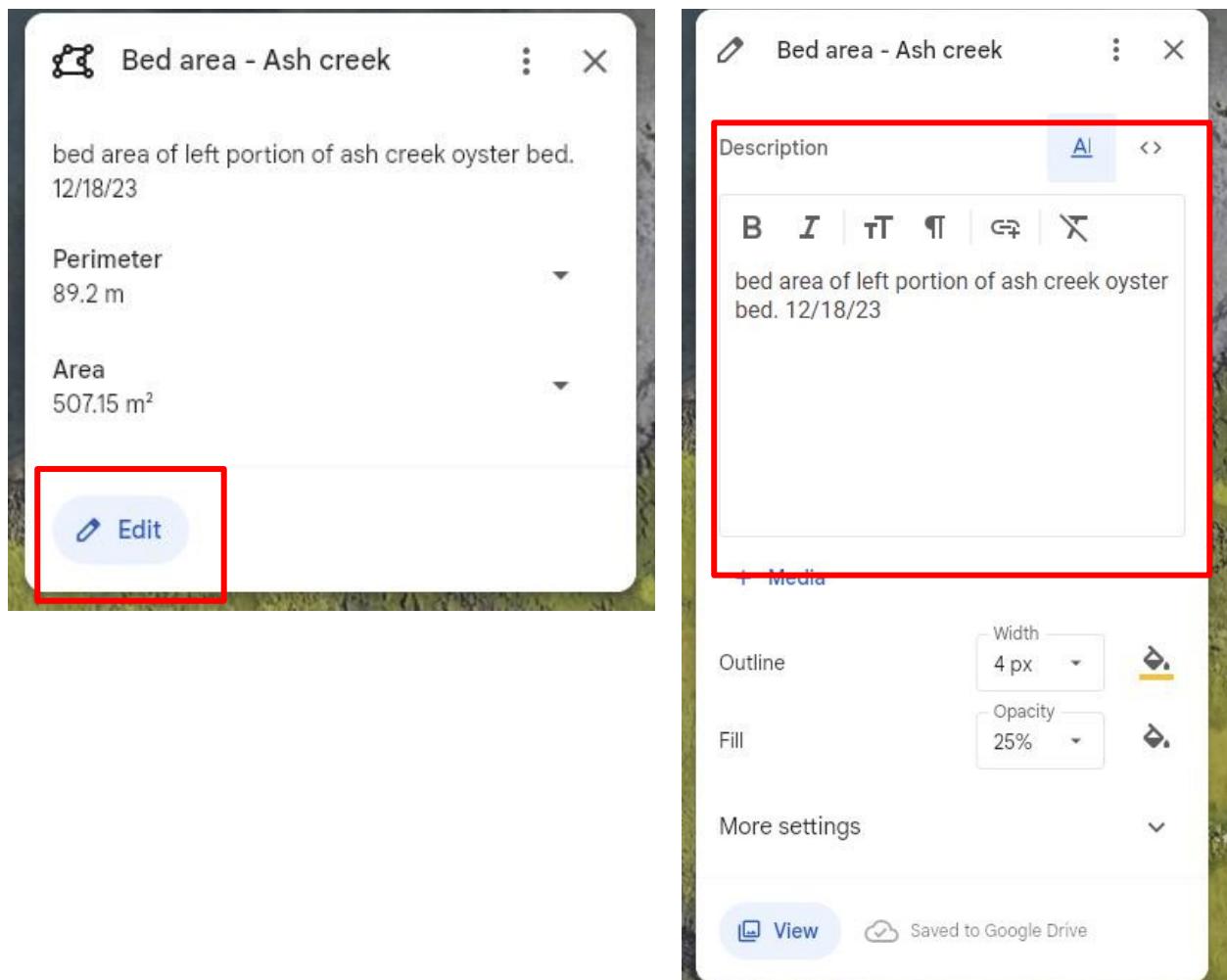


Figure 12: Depiction of how to edit the saved measurement and description.

6. Site specific guidelines

- a. Ash Creek has two bed sections (a right and left reef area). The bed sections are identified as right and left based on the view from entering the access point. Both sections should be measured for area and added together for total reef area (Figure 13).
 - i. If new areas of the bed are added via restoration practices, include the new region in the measurement (may change annually due to restoration activities).



Figure 13: Depiction of the right and left sections of Ash Creek, Fairfield CT oyster bed.

- b. Fence Creek oyster bed includes a former lease site and the natural bed continues under Seaview Ave. Bridge and out towards the ocean. The bed area measurements should only include the start of the bridge on the lease side, under the bridge and out towards the ocean. *Do not include the lease site as we are not sampling or studying population dynamics on that section to avoid damaging that part of the reef.* (Figure 14).



Figure 14: Depiction of where the Fence Creek, Madison CT oyster bed spans.

END OF SOP

Appendix C – Rugosity Measurements SOP

1 Purpose

The purpose of this SOP is to provide concise guidance and methodology on how to measure oyster bed rugosity for intertidal and subtidal reefs to determine surface complexity which impacts productivity, larval recruitment, water flow dynamics, and trophic interactions.

2 Scope

This SOP is pertaining to the EPA Long Island Sound Study funded Oyster Health project where oyster bed rugosity will be used to characterize complexity for each oyster reef as part of biannual population surveys.

3 Definitions/Acronyms

4 Safety Precautions

All survey team members will wear appropriate clothing dependent on weather conditions including but not limited to waders, rubber boots or protective footwear, gloves, hats, sunglasses, long sleeve shirts, and pants. Team members will wash hands thoroughly after each sampling day. A first aid kit will be present for any injury. Extra water will be provided to avoid dehydration or heat stroke. Team members will take regular breaks when needed.

Exercise weather-appropriate field safety measures by monitoring conditions before and during the trip. Do not perform fieldwork during dangerous conditions (e.g. lightning, extreme winds, extreme floods). Do not visit field sites alone (use buddy system). Inform PIs of dates and times of fieldwork. Confirm safe return to the lab. At intertidal sites, perform procedures during low tide. At subtidal sites, divers are to follow NOAA diving regulations according to the instructions of the lab diving coordinator (barry.smith@noaa.gov).

5 Supplies/Materials

1. Fine-link chain attached to U-hook (intertidal)
2. Fine-link chain attached to weighted quadrat (subtidal)
3. Measuring tape
4. Waterproof datasheets
5. Digital datasheets
6. Mechanical pencils

6 Equipment

1. Field tablet

7 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, field notebooks will include a checklist of all data that needs to be recorded during each visit. All datasheets will be screenshotted as back up in the event data is lost before connecting to the network.

8 Procedures

Note: Methods vary slightly between intertidal and subtidal sites and will be described below. Rugosity measurements are not associated with quadrats excavated for density.

1. Measurements will be taken from 3-4 randomly selected locations on the oyster bed.
2. Intertidal Sites
 - a. Mark the start of the rugosity measurement by inserting the U hook attached to the fine-link chain into the oyster bed in a secure position (Figure 1).
 - b. Lay the fine-link chain located in the field kit to contour the surface of the oyster bed along a straight line. This chain has a measurement of **229 centimeters** (fixed chain length). When contouring be sure not to intentionally shove parts of the chain into overhung crevices (Figures 1 and 2). *Be sure that the entirety of the chain is used to contour to keep measurements and calculations precise and consistent.*
 - c. Mark the place where the chain ends with a red flag to guide measurements
 - d. Using the measuring tape, measure to the nearest 0.5 centimeter the straight-line distance between the beginning and end points of the chain created by the contoured chain line to the nearest centimeter (measured contoured chain length, Figure 1).

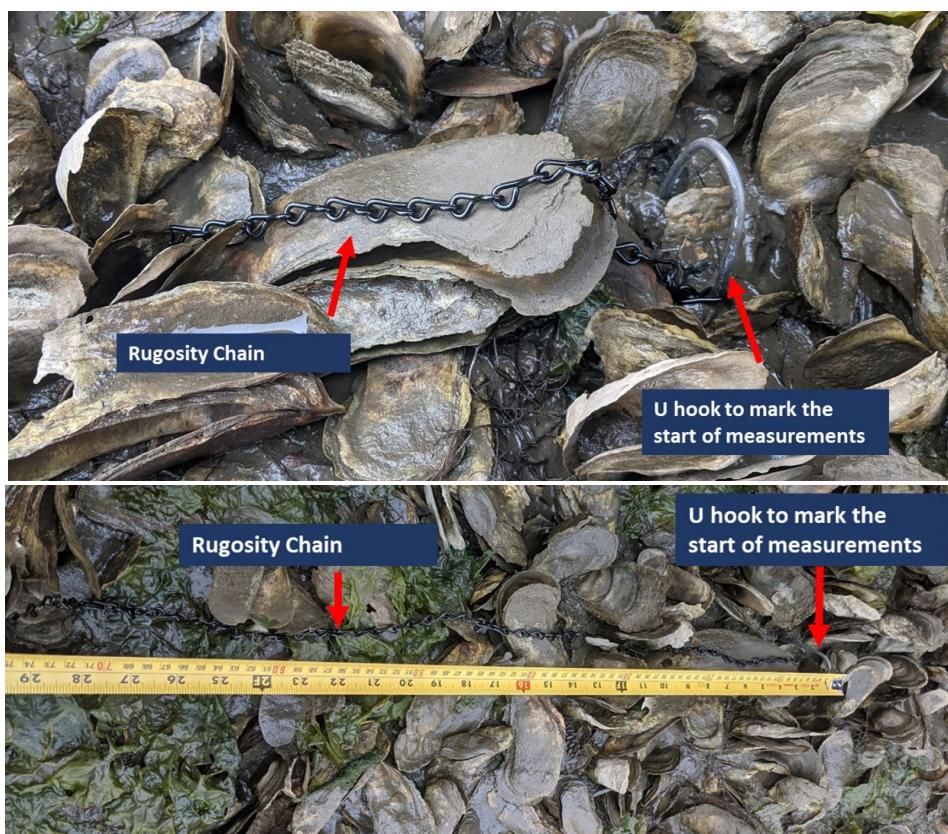


Figure 1: Image of the rugosity chain connected to the secured U hook on an oyster reef (top) and schematic showing method of measuring the contoured chain distance (bottom). Note that the chain is conformed to the surface of the oyster bed without pushing the chain into overhang crevices.

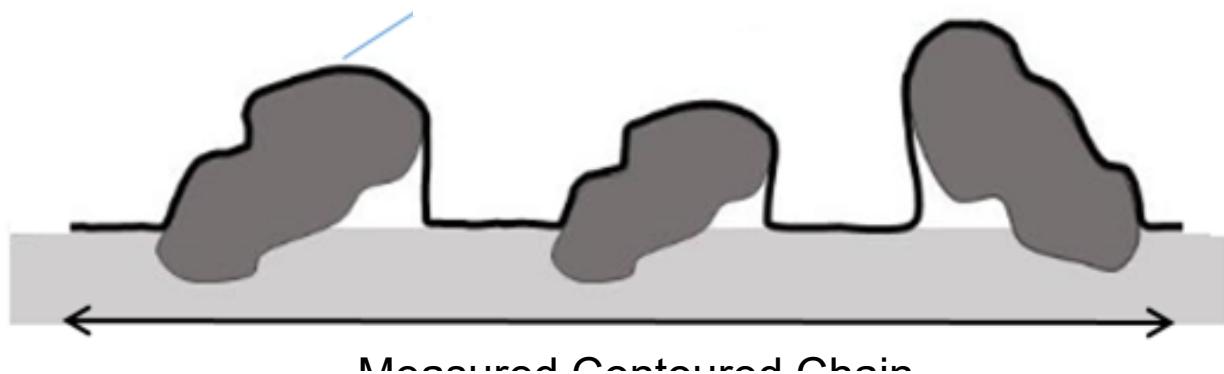


Figure 2: Schematic showing method of measuring oyster bed rugosity. A fine-link chain will be conformed to the surface of the oyster bed without pushing the chain into overhang crevices. Rugosity will be calculated as the measured contoured chain length divided by the fixed chain length (Janiak, 2021).

3. For subtidal sites, the fine link chain will be attached to a weighted quadrat to assist divers to maintain control of the chain underwater and in currents (Figure 3).
 - a. Lay the chain diagonally across the quadrat so that the chain falls into all the crevices.
 - b. Mark the place where the chain meets the diagonal corner of the quadrat with a white zip tie. The diagonal line of the quadrats is a known distance (fixed diagonal length, 35cm) and is marked with a small black zip tie (Figure 3).
 - c. Using the measuring tape, measure to the nearest 0.5 centimeter the straight-line distance between the attachment point and the white zip tie marker to the nearest centimeter (Figure 3). This is the measured diagonal length.
4. Calculating rugosity
 - a. Intertidal sites

$$\text{Rugosity (intertidal)} = \frac{\text{Measured contoured chain length}}{\text{Fixed chain length} *}$$

*The fixed chain length for intertidal sites = 229cm

- b. Subtidal sites

$$\text{Rugosity (subtidal)} = \frac{\text{Fixed diagonal length} *}{\text{Measured diagonal length}}$$

*The fixed diagonal length using a 25 x 25 cm quadrat = 35 cm

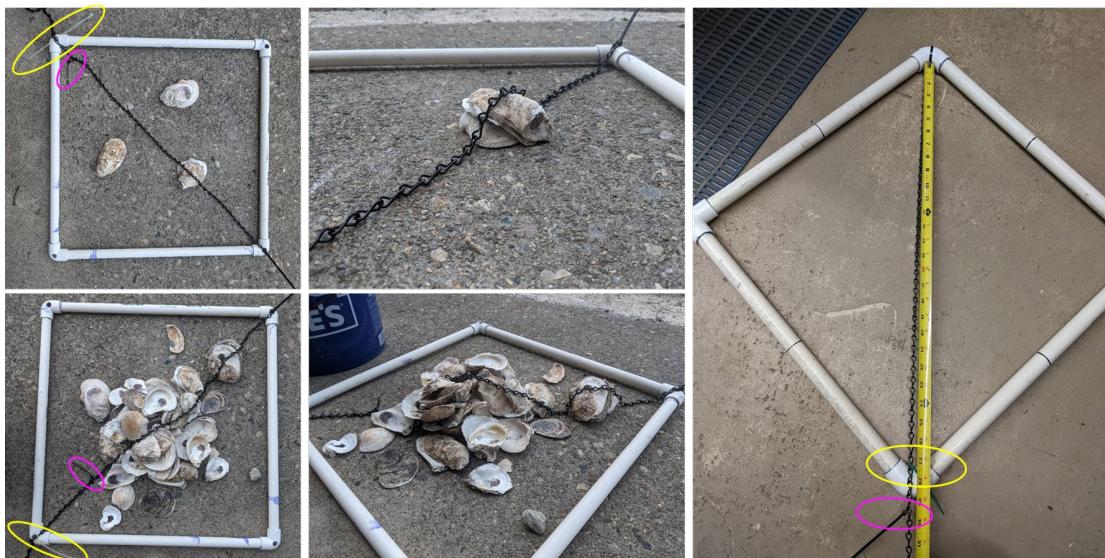


Figure 3: Example of rugosity measurements on low profile and small mound oyster beds. Pink circles mark the length of the chain taught along the diagonal of the quadrat (fixed diagonal length, marked by a black zip tie) and yellow circles mark added zip ties (measured diagonal length). Divers will mark the chain, and the distance between the attachment point and the added zip ties will be measured by the shore team.

- c. The rugosity index will be between 0 and 1, where oyster beds with values closer to 1 demonstrate less complex surfaces (the contoured chain distance is closer to the fixed length). Values closer to 0 indicate a more complex surface (contoured chain covers a shorter distance).
- 5. Values should be recorded in the appropriate datasheet based on site. Example data sheet “characteristics”. Divers will record on a PVC sleeve with waterproof paper.

9 References

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

END OF SOP

Appendix D – Oyster Bed Area, Subtidal SOP

1 Purpose

The purpose of this SOP is to provide concise guidance and methodology on how to measure oyster bed area for subtidal sites.

2 Scope

This SOP is pertaining to the EPA Long Island Sound Study funded Oyster Health project where oyster bed area will be used to characterize the size of the oyster bed at each oyster site as part of biannual population surveys.

3 Definitions/Acronyms

4 Safety Precautions

All survey team members will wear appropriate clothing dependent on weather conditions including, but not limited to waders, rubber boots or protective footwear, gloves, hats, sunglasses, long sleeve shirts and pants. Team members will wash hands thoroughly after each sampling day. A first aid kit will be present for any injury. Extra water will be provided to avoid dehydration or heat stroke. Team members will take regular breaks when needed.

Exercise weather-appropriate field safety measures by monitoring conditions before and during the trip. Do not perform fieldwork during dangerous conditions (e.g. lightning, extreme winds, extreme floods). Do not visit field sites alone (use buddy system). Inform PIs of dates and times of fieldwork. Confirm safe return to the lab. At subtidal sites, divers are to follow NOAA diving regulations according to the instructions of the lab diving coordinator (barry.smith@noaa.gov).

5 Supplies/Materials

1. Field Measuring Tape
2. Waterproof paper attached to PVC dive sleeve
3. Number 2 wood pencil
4. Mechanical pencils
5. Extra datasheets
6. Digital datasheets

6 Equipment

1. Field tablet
2. GPS tracker

7 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. All datasheets will be screenshotted as back up in the event data is lost before connecting to the network.

8 Procedures

1. *To determine the area of an oyster bed, methods described in Janiak (2021) will be used.*
 - a. With one diver on either end, place a weighted measuring line (Figure 1) along the longest axis of the oyster bed starting at the edge or perimeter.
 - i. The perimeter is defined as the continuous edge where live or dead shells make up ~25% of the substrate (Janiak, 2021).
 - b. Divers will record the total length of the oyster bed on the dive sleeve datasheet for the site.
 - c. Divers will measure and record the width of the bed at 3–6 random locations covering the length of the longest axis line (Figure 1).
 - d. The shore team will transcribe these data onto the appropriate data sheet on the tablet. Example data sheet “characteristics”
 - e. Area of the oyster bed will be calculated from the resulting polygon created in Google Earth following the Oyster Bed Area, Intertidal SOPI.

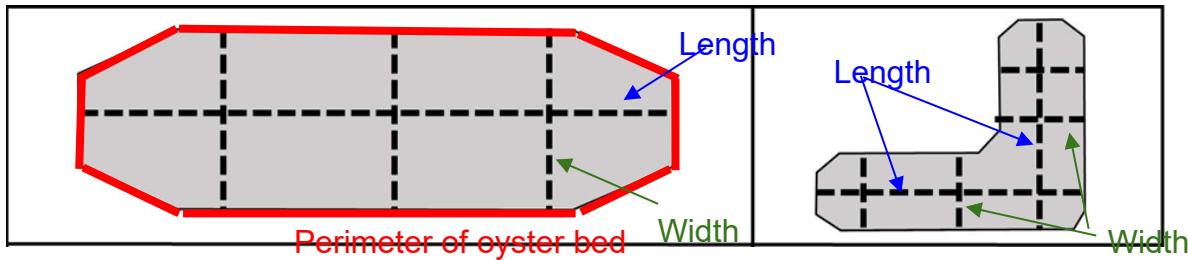


Figure 1: Example schematic of oyster bed area measurements.

9 References

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

END OF SOP

Appendix E – Oyster Bed Density & Size Distribution Using Quadrats SOP

1 Purpose

The purpose of this SOP is to provide concise guidance and methodology on how to sample quadrats for intertidal oyster population surveys to determine density and size class distribution.

2 Scope

This SOP is pertaining to the EPA Long Island Sound Study funded Oyster Health project where quadrats will be used to quantify live oyster densities, mortality ratios, and size distributions as part of biannual oyster bed surveys included in the project efforts. For subtidal oyster beds, divers will conduct the quadrat excavations and transport them to a shore team for measurements.

3 Definitions/Acronyms

spat = recently settled juvenile oyster

4 Safety Precautions

All survey team members will wear appropriate clothing dependent on weather conditions including but not limited to waders, rubber boots or protective footwear, gloves, hats, sunglasses, long sleeve shirts, and pants. Thick protective gloves (e.g garden gloves) should be worn when handling oysters. Team members will wash hands thoroughly after each sampling day. A first aid kit will be present for any injury. Extra water will be provided to avoid dehydration or heat stroke. Team members will take regular breaks when needed.

Exercise weather-appropriate field safety measures by monitoring conditions before and during the trip. Do not perform fieldwork during dangerous conditions (e.g. lightning, extreme winds, extreme floods). Do not visit field sites alone (use buddy system). Inform PIs of dates and times of fieldwork. Confirm safe return to the lab to PIs or Project Leads. At intertidal sites, perform procedures during low tide. At subtidal sites, divers are to follow NOAA diving regulations according to the instructions of the lab diving coordinator (Barry Smith).

5 Supplies/Materials

- Field Backpack (See Appendix A)
- Folding chair and tables
- Gray bins
- Permits and permission letters
- Spare data sheets (on waterproof paper)
- Waterproof paper
- Mechanical pencils
- Ruler
- Copy of survey instructions
- First aid kit
- Povidone Iodine scrub
- Hand lotion
- Hand sanitizer
- Bug spray
- Sunscreen

- Specimen collection bags
- Laminated SOPs
- Cooler with ice
- Buckets (x8)
- 2-3 Quadrats, 25 x 25 cm
- Field Gloves
- Calipers (plastic for back up)
- Tool box
- Waders
- Boots
- Scrubbing brushes
- Shucking knives
- Tally counters
- Gray trays (x6)
- Towel
- Kneeling pads
- Diving:
 - Towable dive flag
 - Diver collection bags (x2)
 - Personal Flotation Device (PFD) for row boat
 - Wire cutters on lanyard
 - Large rubber bands
 - Dive tanks
 - AED
 - Oxygen supply

6 Equipment

- Field tablet (charged)
- Tablet charger and spare battery
- Digital bluetooth calipers

7 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, field notebooks will include a checklist of all data that needs to be recorded during each visit. All datasheets will be screenshotted as back up in the event data is lost before connecting to the network.

8 Preparation

1. At the lab:
 - a. Pre-label all collection bags and sorting bins or buckets. Collections bags should have the site name. Sorting bins and bucks should be labeled for the number quadrat (ex: 1-4).
 - b. Load the field vehicle with all equipment and supplies necessary to conduct the surveys using the checklist.
 - c. Make sure all team members have appropriate clothing and gear to proceed with the day (both diving and on land).
 - d. Assign team members tasks for the survey such as excavators and measurers.
 - e. Prepare the data sheets by ensuring the proper site labels, dates, and number of quadrats is updated.

2. On Site:

- a. Before starting the survey, a measuring station should be set up. This includes setting up a folding table with all supplies and bins needed to sort and measure oysters. Each station should include two pre-labeled gray bins, a bluetooth digital caliper, a tablet, a bucket of seawater, and field gloves of appropriate size.
- b. Make sure that all calipers are working and connected to the designated tablets. The calipers are labeled #1 and #2 and are matched with a like-numbered tablet.
 - i. Enter the bluetooth settings on the tablet.
 - ii. Turn the caliper on (Figure 1). Hold the top right button in until there is a blinking bluetooth symbol in the top left corner of the screen.
 - iii. The caliper name should appear in the devices list in the bluetooth connection settings.
 - iv. Click on the appropriate caliper device (Caliper 1 or Caliper 2).
 - v. The caliper should now be connected to the tablet and ready for use. To check connection, go into the appropriate datasheet and do a few test measurements before beginning the survey.
 - vi. **Note that both calipers will have trouble connecting to their designated tablet if within close proximity. Try connecting a few feet away from each other.**

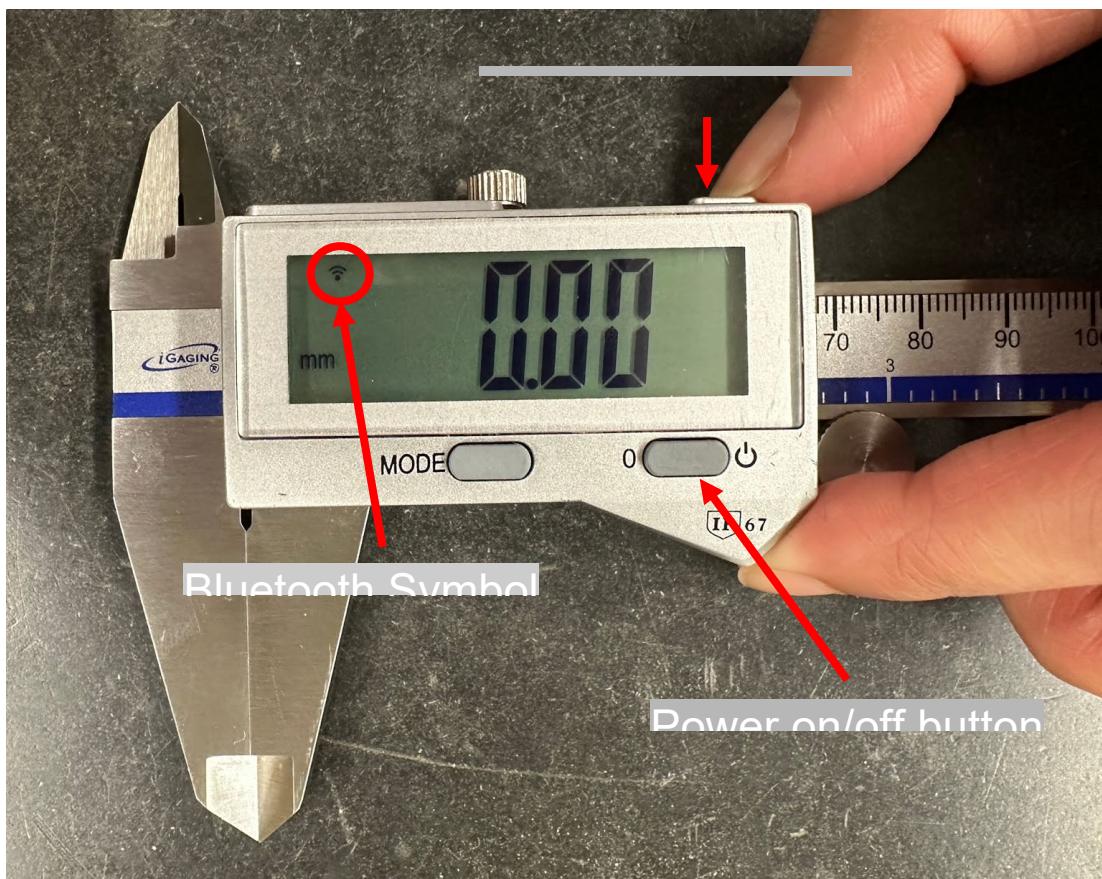


Figure 1: Image of digital bluetooth enabled calipers. Arrows point to both the power on/off and bluetooth connect buttons as well as the bluetooth symbol. The bluetooth symbol flashes when searching for an enabled device and will appear static when connected.

9 Procedures

Note: techniques for quadrat collection may vary slightly between subtidal and intertidal sites. Variations will be specified in instructions.

1. Randomly lay the 25cm x 25cm quadrat within the target zone on the bed (See Appendix B for maps of reef locations). At subtidal locations, 3-4 quadrats will be taken from the oyster bed (Janiak, 2021). To account for the greater variation in reef composition and structure at intertidal locations, 5-6 quadrats will be collected.
 - a. All quadrats should be either weighted or have holes drilled to allow water to fill and sink the quadrat. All quadrats for subtidal sites will be weighted.
2. Percent coverage:
 - a. Estimate percent coverage within the quadrat's inner perimeter (inside of the quadrat square). This will be done for each quadrat.
 - i. Coverage includes all live, gapers, and box oysters, but not random loose half shells that may have washed up (Table 1).
 - ii. Coverage options are: 26-50%, 51-75%, 76-99%, and 100%. At these sites, if a quadrat has 0-25% coverage, then it is too far into the mud flat or bottom substrate and off of the oyster bed. Examples are shown in Figure 2.
 - b. Record percent cover in the appropriate quadrat datasheet.

Table 1. Description of oyster categories that will be assessed for population demographics.

Source: Tarnowski, 2021.

Oyster category	Description
Live	Live oyster
Gaper	Dead or moribund oyster with gaping valves and tissue still present.
Box	Pairs of empty shells joined together by their hinge ligaments.

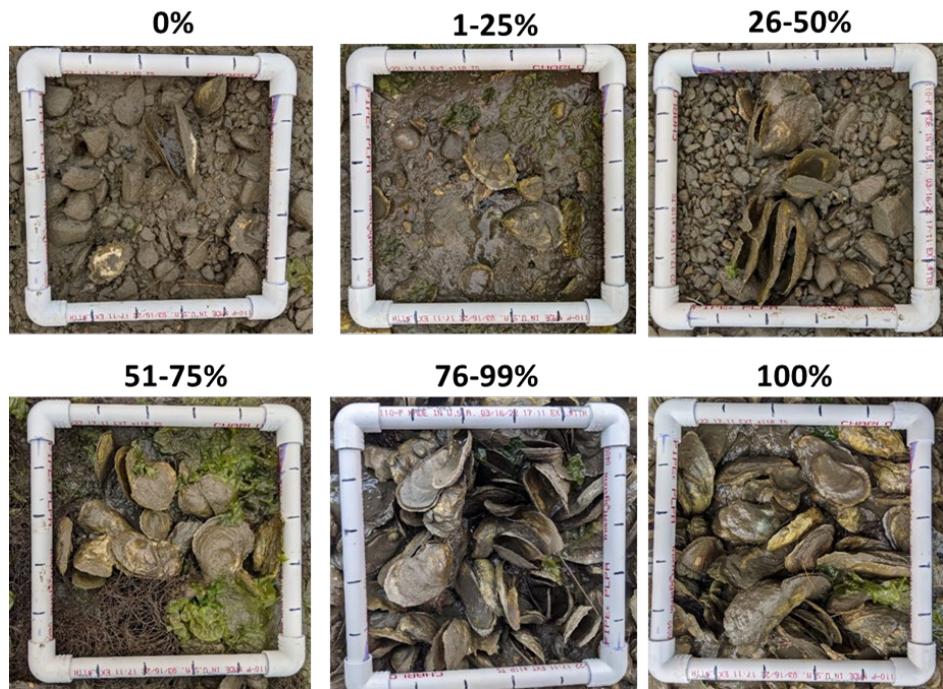


Figure 2: Examples of estimated oyster coverage.

3. Excavating the quadrat:
 - a. Wearing protective gloves, excavate all oysters (Live, Gapers, and Box; see below) from inside the quadrat. (Figure 3 - Left Image) **Oyster clumps should be preserved as well as possible to avoid killing animals and disrupting the physical structure.**
 - b. If $\geq 50\%$ of the oyster is within the inner perimeter of the quadrat frame, it is counted as “in the quadrat.” If $< 50\%$ of the oyster is outside of the inner quadrat perimeter (shaded green in Figure 4) it is counted as “out of the quadrat.”
 - i. There will be difficult calls from time to time (eg. the question mark in the bottom right quadrat of Figure 4). The quadrats do not lay perfectly and oysters are not flat on the bottom. In these cases it is important to have confidence in your best judgment and remain consistent in your calls among quadrats.
 - c. All oysters counted as “in the quadrat” should be moved to a labeled bucket or gray bin for height measurements (Figure 3 - Right Image). Divers will have varying colored collection bags that are pre-labeled for specific quadrats to fill with excavated oysters. These bags will be brought to shore for the measuring team to sort.
 - d. Take the bucket or bin to the measuring station for designated team members to begin processing. Divers will bring collection bags to shore.



Figure 3: Left image - Excavation of a quadrat. Right image - Excavated oysters in gray bins for sorting while measuring. All oysters begin in the first bin and are moved to the 2nd bin as they are counted and measured.

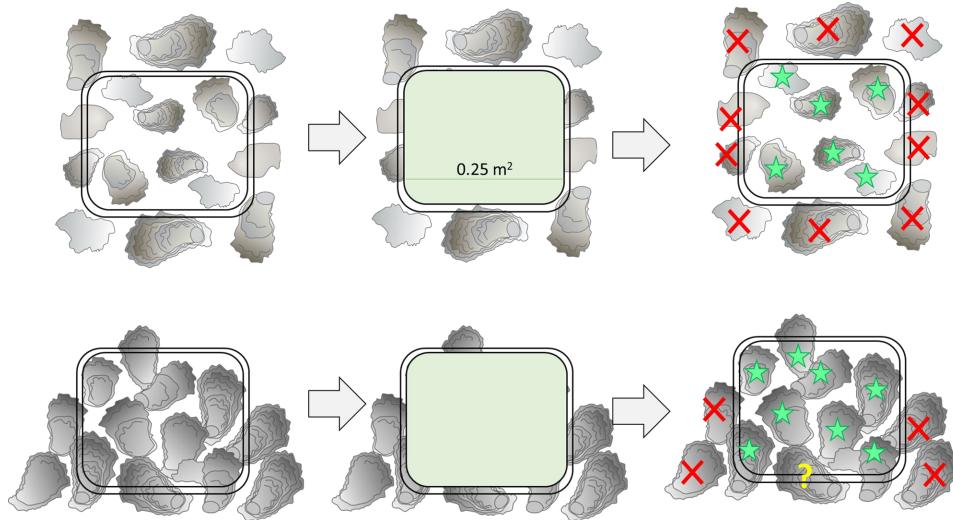


Figure 4: Examples of oysters to be considered inside (green stars) and outside (red X's) of the quadrat. Only the inner perimeter of the frame counts as inside the quadrat (green shaded box).

4. Determining Survival:

- a. The survival status of all excavated oysters should be recorded alongside the shell measurements.
- b. Mud oysters can sometimes appear as though they are live oysters. In particularly muddy regions, shaking the oyster clumps in a bucket of seawater can help to open mud oysters and dislodge the mud suctioning them closed
- c. Oysters can be classified as Live, Gaper, or Box (Table 1; Figure 5).

- i. Live oysters will remain fully closed when removed from the water and during handling.
- ii. Boxes are fully open with no tissue remaining inside the shell.
- iii. Gapers are open with partial or all tissue remaining (in some cases only the adductor muscle will remain) indicating a recent mortality.
- d. *Only oysters where both shells are articulated at the hinge should be counted. Disarticulated single shells are not included in the survival counts and shell measurements.*

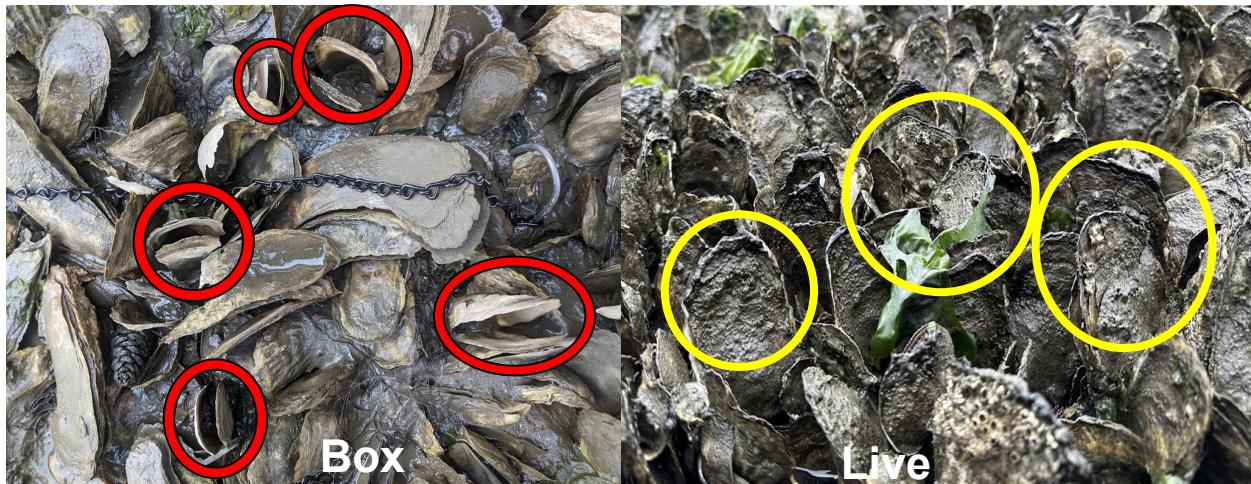


Figure 5: Examples of box and live oysters. Live oysters will remain fully closed when handled, boxes are fully open with no tissue remaining inside the shell, and gapers are open with partial or all tissue remaining.

5. Shell Measurements

- a. Using a bucket of seawater, rinse oyster clumps to remove all mud from oysters prior to measuring to ensure that no animal is being missed.
- b. As shown in Figure 6, using the digital bluetooth calipers, measure the shell height of up to 100 oysters in the quadrat. Record shell height in the column reflecting the survival status (Live, Box, Gaper Example data sheet). Remaining oysters in each quadrat (>100) will be counted, but not measured for each category (Live, Box, Gaper) for proper mortality estimates. *Ensure that each measurement is in the proper column or row in the spreadsheet (Live, Box, Gaper) and the caliper is recording the numbers properly.*
 - i. Shell height is measured from the hinge tip to the bill in millimeters and to the nearest decimal (Figure 6).
 - ii. Oysters of all ages and sizes will be measured including recently settled oyster spat (Figure 8).
 - 1. Recently settled spat are likely to be prevalent during the fall surveys and can be difficult to spot. Take care to check for small

- oysters (<10mm) and clean the oyster in the bucket of seawater as needed to improve visuals.
- iii. Other juvenile shellfish can be mistaken for oysters (Figures 7 & 8), so be sure that detailed checks are made during the assessment.
 - iv. Oysters are often heavily clumped together. Try to identify the hinge and bill of each oyster to measure. Calipers will have to be fit into crevices.
6. After measurements are complete, collect 3-4 adult oysters (80mm -120mm) from each quadrat in the pre-labeled collection bag for lab processing. The remaining individuals (to reach a total of 33 oysters) should be randomly selected from across the reef. Sampled oysters should be placed on ice to be brought back to the lab. See the Monthly Sample Collection SOP for more details. Return the remaining animals to the excavated region to reduce disturbance to the reef.

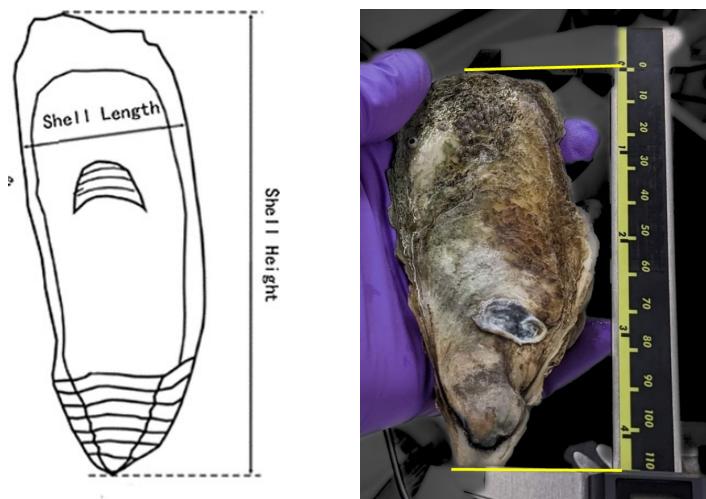


Figure 6: Collecting shell measurements with calipers. Schematic showing height and length measurement landmarks (left). Shell height measurement on live oyster (right).



Figure 7: Limpets can sometimes be mistaken for juvenile oysters. Yellow circles in the photos indicate limpets on oyster shells.



Figure 8: There are often many overlapping juvenile oysters on a single loose shell or live adult oyster. In some cases, they are completely growing on top of each other (eg. pink circle) making it difficult to get good measurements without killing the overlaying oysters to break up the clump.

7. Take down & clean up

- Ensure that all datasheets are complete. Screenshot the datasheets as backup in the event data is lost before connecting to the network.
- Return oysters from excavated quadrats to the oyster bed where they were collected (minus those selected for lab processing). For subtidal sites, divers will return to the general area or the shore team will dump oysters back into the water.

- c. Rinse equipment off with water to remove excess mud to transport back to the lab. Ensure that no animals are residing in the buckets or bins.
- d. Using the checklist, repack equipment into the field vehicle to ensure everything is accounted for and not left behind.
- e. Wash hands or use hand sanitizer after oyster collections. Ensure that no one has any cuts or injuries.
 - i. *At Fence Creek, due to high bacterial load, use the Povidone Iodine scrub to cover your hands/arms and allow it to sit for 1 minute on intact skin or a minimum of 5 minutes (maximum of 10 minutes) on any cuts or wounds. If you are prone to skin irritation with povidone iodine scrub, discontinue/avoid use. Be sure to remove any clothing or jewelry that you do not want to become stained. Rinse thoroughly with water.*
 - ii. *Immediately follow the above procedure for povidone iodine application if a cut is acquired while at any site.*

10 Lab return and clean up

1. All equipment should be hosed down with fresh water including bins, buckets, tools, tables, and field clothing.
2. Metal tools (eg. clickers, calipers etc.) should be sprayed with WD-40, with focus on the hinges to prevent rusting.
3. Waders and gloves should be hung upside down to dry.
4. All equipment should be put away in their designated area in the Oyster Health Field gear area.
5. Oyster samples should be immediately taken to Rm 29 to be processed or placed in the fridge.
6. Divers are responsible for their own gear.
7. Make sure all notes have been updated in the appropriate field notebooks.

11 Calculating mortality indices (adapted from Ford et al. 2006):

Example data sheet has the following calculations built in for a quick assessment of the data collected.

$$\text{Total mortality (\%)} = \frac{\text{number of gapers + box / quadrat}}{\text{number of gapers + box + live oysters / quadrat}} \times 100$$

$$\text{Gaper mortality (\%)} = \frac{\text{number of gapers / quadrat}}{\text{number of gapers + live oysters / quadrat}} \times 100$$

$$\# \text{ Live oysters per m}^2 = \text{number of live oysters counted/quadrat} \times 16$$

12 References

- 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>
- Janiak, D., 2021. MarineGEO Oyster Reef Habitat Monitoring Protocol. Tennenbaum Marine Observatories Network, MarineGEO, Smithsonian Institution.
- Tarnowski, M., 2021. Maryland Oyster Population Status Report: 2021 Fall Survey. Maryland Department of Natural Resources, Annapolis MD.
- Whitlock, M.C., Schluter, D., 2019. The analysis of biological data, Third edition. ed. Macmillan Learning, New York.

13 Appendices

- A. What goes into the field backpack?
 - a. Tablets
 - b. Digital Calipers
 - c. Extra gloves
 - d. Extra zip ties
 - e. Field notebook
 - f. Permits and permission letters
 - g. Spare data sheets (on waterproof paper)
 - h. Waterproof paper
 - i. Mechanical pencils
 - j. Ruler
 - k. Copy of survey instructions
 - l. First aid kit
 - m. Povidone Iodine scrub
 - n. Hand lotion
 - o. Hand sanitizer
 - p. Bug spray
 - q. Sunscreen
- B. Maps of sites

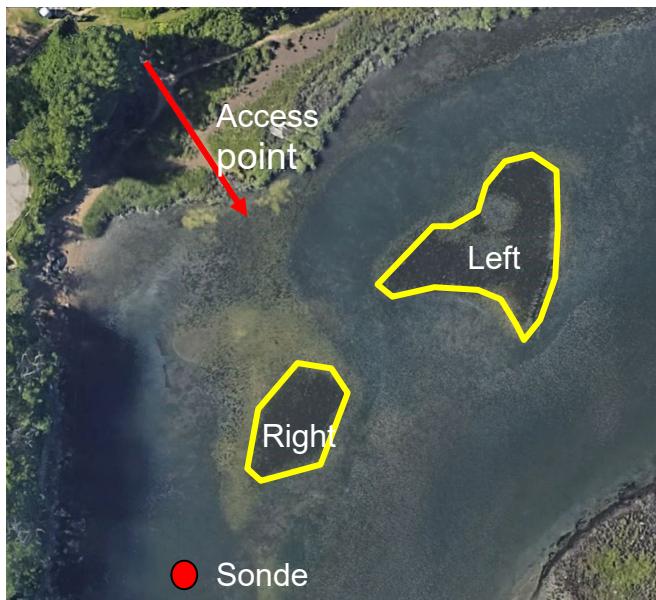


Figure 1: Aerial map of Ash Creek. Yellow perimeter indicates where the oyster bed resides.



Figure 2: Aerial map of Fence Creek. Yellow perimeter indicating where the oyster bed resides.



Figure 3: Aerial map of Gold Star Beach oyster bed general location off of the dock.

END OF SOP

Appendix F – Recruitment SOP

1 Purpose

The purpose of this SOP is to provide concise guidance and methodology for deploying and processing shell stringers at intertidal and subtidal oyster beds to monitor oyster recruitment and reproduction.

2 Scope

This SOP is pertaining to the EPA Long Island Sound Study funded Oyster Health Project that is incorporating recruitment and reproductive reevaluations to understand oyster population health within Long Island Sound.

3 Definitions/Acronyms

spat = juvenile oyster (shell length < 40 mm)

4 Reagents/Media

REScue, 1:16 dilution in water ([REScue Concentrate](#), 4.25% accelerated hydrogen peroxide)

5 Supplies/Equipment

- Drill press
- Magnifier lamp
- Calipers
- Clicker counters
- Scrub brush
- Shucking gloves / protective work gloves
- Weighted galvanized wire
- Washers
- Pliers
- Wire clippers
- Cured oyster shells (6 month minimum)
- PVC
- Rebar

6 Safety Precautions

All collection team members will wear appropriate clothing dependent on weather conditions including but not limited to waders, rubber boots or protective footwear, gloves, hats, sunglasses, long sleeve shirts, and pants. Thick protective gloves (e.g garden gloves) should be worn when handling oysters. Team members will wash hands thoroughly after field trips end. A first aid kit will be present for any injury. Extra water will be provided to avoid dehydration or heat stroke. Team members will take regular breaks when needed.

Exercise weather-appropriate field safety measures by monitoring conditions before and during the trip. Do not perform fieldwork during dangerous conditions (e.g. lightning, extreme winds, extreme floods). Do not visit field sites alone (use buddy system). Inform PIs of dates and times of fieldwork. Confirm safe return to the lab. At intertidal sites, perform procedures during low tide. At subtidal sites, divers are to follow NOAA diving regulations according to the instructions of the lab diving coordinator (Barry Smith barry.smith@noaa.gov).

Laboratory members will wear safety gear including gloves and goggles when cleaning cured shells in preparation to build stringers and while using the drill press. Members will follow standard laboratory procedures in the event of an accident and all team 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 cabinet doors.

7 Laboratory Quality Control

Cured shells are sourced from the Fairfield Shell recycling program and will have a minimum of 6 months curing duration on land to ensure there are no spread of disease or invasive species with the shells.

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 among members prior to starting to ensure uniformity. Duplicate reads of 10% of the shells should be conducted as a quality assurance step and discrepancies discussed while observing the shells. To ensure completeness, field notebooks will include a checklist of all data that needs to be recorded during each visit. All datasheets will be screenshotted as back up in the event data is lost before connecting to the network. **Any notes should be recorded in the site specific field notebook and recruitment notes file.**

8 Procedures

1. Prior to field deployment, prepare shell stringers.

- a. Each oyster bed site will need 6 shell stringers, containing 6 cured oyster shells per stringer.
- b. To make shell stringers for one site, obtain 36 cured oyster shells (100–120 mm in height). The shells should be smooth with no spat scars or remnants of biofouling organisms such as barnacles or boring sponge. This will be beneficial when counting spat. If the shell is in good condition, spat scars and biofouling can be removed with a shucking knife to create a smooth surface.
- c. Using a drill press (located in the workshop - Building 3 of the Milford Lab), drill a single hole in the center of the shell. Be sure to wear appropriate protective gear such as gloves and goggles when using the drill press.
 - i. Must have proper training and approval before operating the drill press (*details forthcoming*)
- d. Once holes have been drilled in all oyster shells (36 total per site), they can be strung (6 per stringer) using weighted galvanized wire and washers (Figure 1). The shells should be oriented with the inner surface (concave) facing down.



Figure 1: Depiction of washers and weighted galvanized wire.

- e. Once the shells have been strung they can be attached to a PVC T-bar by looping the wire through pre-drilled holes in the PVC (Figure 2). Loop the galvanized wire through the holes on either side of the T bar and twist many times to securely fasten to the structure. A NOAA tag should be attached to the stringers with contact information for the Lead Field Scientist.



Figure 2: Example of shell stringer attached to T-bar. (Photo from [Florida Fish and Wildlife Conservation Commission](#))

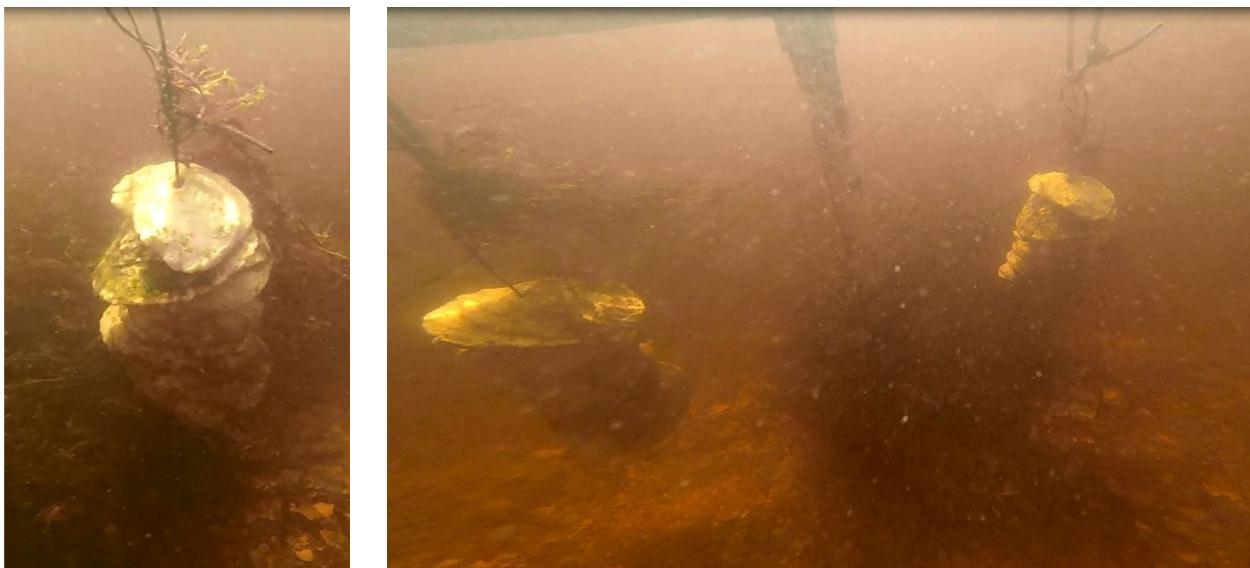


Figure 3: Underwater photograph of oyster shell stringers suspended from a PVC T-bar frame, as used by McFarland et al. (2022).

2. Deployment

- a. At each site and each deployment, 6 shell stringers, each containing 6 cured oyster shells, will be deployed for spat collection and quantification.
- b. Stringers will be deployed at three time points within a sample year, in June and August (totaling 18 stringers per site per season).
- c. The stringers will be deployed along the growing edge of the oyster bed.
- d. Stringers should be suspended at least 5 cm above the seabed on their T-bar frames. This will aid in avoiding predation on settled spat (Figure 3).

- e. Site specific instructions:
 - i. **Intertidal sites (Ash Creek and Fence Creek):** PVC T-bars holding the shell stringers are securely deployed using rebar on the oyster bed. Rebar should be driven deep enough to ensure it will remain secure in the seabed. The PVC will have holes drilled through it to add zip-ties for additional security.
 - 1. At Ash Creek the stringers are deployed on the fringe of the reef on the far side of the left reef bed (Figure 4).
 - 2. At Fence Creek the stringers are deployed at the base of the bridge on the ‘lease’ side (Figure 5).



Figure 4: Shell stringer deployment location at Ash Creek, Fairfield, CT.



Figure 5: Shell stringer deployment location at Fence Creek, Madison, CT.

- ii. **Subtidal- Gold Star Beach:** Shell stringers will be deployed on T-bars secured to cement blocks deployed alongside the oyster mounds (Figure 6). Shell stringers will be deployed and retrieved by divers.



Figure 6: Shell stringer structure deployment for Goldstar Beach, Huntington Bay, NY.

3. Bimonthly at each site, all shell stringers will be retrieved and new shell stringers deployed for spat collection and quantification.
 - a. ***Therefore, June deployment will be assessed in August and August deployment will be assessed in October.***
 - a. Retrieved shell stringers will be removed from the T-bar by cutting the galvanized wire with wire clippers and strings will be held in a bucket of seawater or in a cooler on ice with their labeled tags for the site until ready to return to the lab.
Note: Shells should remain in order and on their individual strings with tags until counted in the lab.
 - i. At the end of the field season, the PVC T-bar from the final deployment should be returned to the lab. The rebar can be left in place for the following season.
 - b. Stringers should be transported to the lab for analysis on ice in the cooler.
 - c. Once the stringers have been returned to the lab, they can be disassembled (being careful to keep the shells in order and replicates separated). Lightly rinse each individual shell with fresh water, and gently scrub to remove any fouling organisms such as algae or mud to increase visibility of settled spat (Figure 7).



Figure 7: Depiction of fouled oyster shell stringer ready to be disassembled and cleaned.

- d. Cleaned shells should be placed on a tray with an assigned shell number (#1-6) for each stringer (Figure 8).



Figure 8: Depiction of shell stringers disassembled, cleaned and placed on a tray with an assigned number.

- e. At the time when recruitment is assessed, oyster spat can be verified under a magnifier lamp. Recruitment will be quantified as the mean number of newly settled oysters per oyster shell (top and bottom), deployment date, and site. Spat counts will be done in duplicate for quality assurance purposes (Figure 9). **Note:**

There are other species that may look like spat such as slipper snails. See Figure 10 to help identify differences.

- f. Counts will be tallied using a clicker counter and measurements of spat will be taken using calipers. Data will be recorded in data sheets.
- g. **Any notes should be recorded in the site specific field notebook and recruitment notes file.**

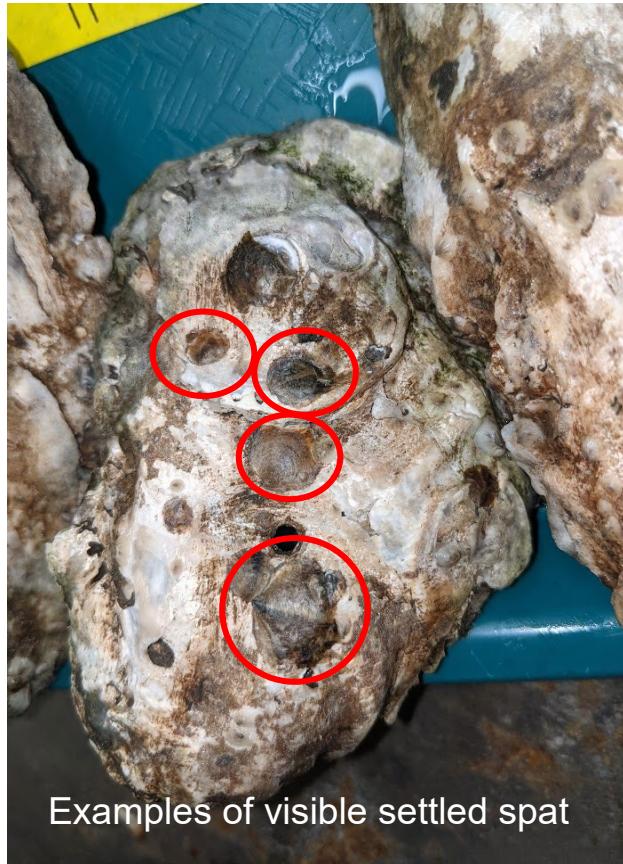


Figure 9: Depiction of counting under a magnifier and examples of visibly settled spat on shell.

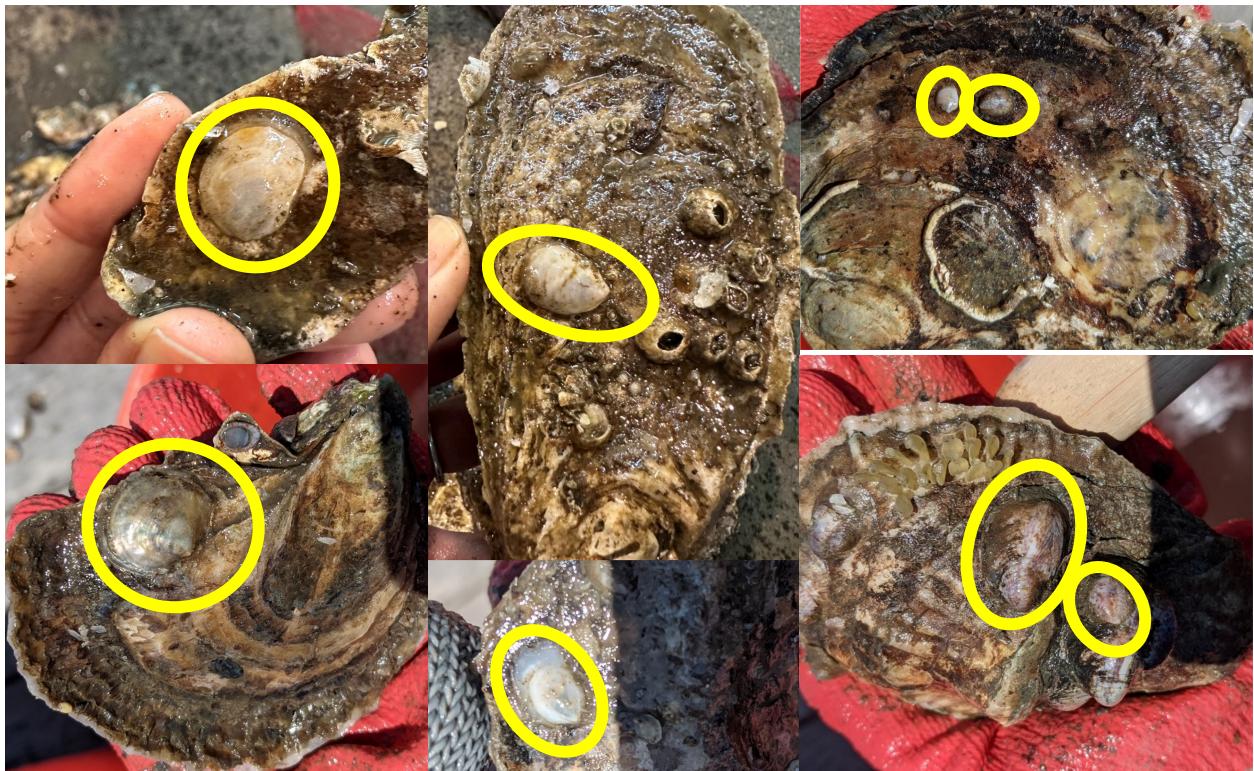


Figure 10: Depiction of slipper snail juveniles attached to shell stringers and oysters.

9 Calculations

$$\text{Top shell count} + \text{Bottom shell count} = \text{Total spat count per shell}$$

$$\frac{\text{Sum of total spat count from 6 individual shells}}{6 \text{ individual shells}} = \text{mean spat count per shell stringer}$$

10 Clean up

1. All oyster shells should be gently scrubbed clean of any biofouling, including the spat, and set to dry outside (~24 hrs). These shells will be used in the following season after 6 months of dry storage.
2. All tissue and growth scraped from the shells should be thrown in the trash and removed from the laboratory at the end of the day.
3. All tools and trays should be washed with soap, dried, and put away.
4. All lab surfaces should be sprayed with disinfectant (REScue, 1:16 dilution in water), allowed to sit for 5 minutes contact time, and wiped down.
5. Inventory of supplies should be taken to prepare for the next sampling period.
6. All laboratory notebooks and data sheets should be updated and checked for quality assurance and control.
7. **Any notes should be recorded in the site specific field notebook and recruitment notes file.**

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

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>

END OF SOP

Appendix G – 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 health and 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
 - Glycerin, Reagent Grade, LabChem, LC148502
 - Formaldehyde, 37% by weight (Histology), Fisher Chemical, F75P-20
 - Ethanol (95%)
 - NaCl [7647-14-5] S-3014. FSigma Chemical Company, St. Louis, MO
 - Deionized water
- 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 or histology safe plastic 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 (1000 μ L, 200 μ L, 50 μ L, 10 μ L, 2 μ L)
- Pipette tips (1000 μ L, 200 μ L, 50 μ L, 10 μ L, 2 μ L)

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 in a fume hood 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

APPENDIX G

- environment. Trash bins containing non-biohazard waste should also be emptied in the facility dumpster in the parking lot. 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. Cut clean paper towel pieces for sampling individual oyster tissues.
 7. 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 with the individual sample ID, following the scheme: MMYY SITE _##
 - i. MMYY refers to the collection month and year
 - ii. SITE is the 4 letter abbreviation for the collection site (ASHC = Ash Creek, FENC = Fence Creek, GOLD = Goldstar 2022 reef, and STAR = Goldstar 2023 reef)
 - iii. ## is the 2 digit individual oyster sample number
 - iv. The histology jar should be labeled with the sample ID set and PI last name. Leave room to add fixative details after samples are added.
 - b. Microcentrifuge tubes will be split into main and duplicate sets.
 - i. The main set will be labeled with permanent marker with the individual animal ID (MMYY SITE _##) on the side, and A/F/G/S## on the lid.
 - ii. The duplicate set 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).
 - iii. The main samples will go into a single freezer box and will be grouped by collection month and state (ie. CT samples from the same month in one box). The duplicate samples will go into a separate freezer box with the same grouping. Freezer boxes should be labeled on the top and side of the bottom with the following information:
 1. Sample IDs of individual oysters included in the set. This is typically 30 oysters.
(MMYY SITE _##-->##; ie. 0423ASHC_01→30)
 2. Tissue type (ie. Oyster Gill, Gut, Rectum)
 3. Main or Duplicate
 4. Preservation type (fresh frozen or 95% EtOH)
 5. PI Name
 8. Prepare any media/reagents needed for processing. Pre-fill duplicate set of microcentrifuge tubes with 95% ethanol for the qPCR samples. Prepare RFTM, (see "RTFM" SOP) and pre-fill labeled culture tubes. Prepare Davidson's fixative (see "Davidson's fixative & Histology slide preparation" SOP).
 9. New electronic data collection sheets should be generated, organized, and dated, and they should be printed or electronically available for data recording. See 'TEMPLATE_TissueProcessing'.
 - a. The template data sheet should be copied and renamed as MMYY SITE _TissueProcessing. This data sheet should be saved in the Google Drive data repository (Project Planning > Data Management > Lab Data > Tissue Processing > Working_Folder).

- b. Cells B2 and C2 should be updated to reflect the collection date and site for the sample set.

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. Prepare a work station by setting up the sterilization station described in step 10.7.a. below. Make sure there are adequate dissection tools, trays, and take inventory of the labeled tubes for RFTM, qPCR ethanol preserved, and histology cassettes. Make sure there are pre-cut foil and paper towels. Obtain the caliper and scale to measure and weigh the animals. Prep the oyster cleaning sink with a 6% bleach solution, brushes, and shucking knives.
3. Using cold freshwater, scrub animals with the bristle brush and rinse animals well. With a shucking knife, remove all fouling organisms such as spat, barnacles, and algae.
4. Scrub hinges with a toothbrush and 6% bleach (diluted 1 part bleach: 10 parts water) to sanitize the area. Use a paper towel to remove excess water and set aside on an individually labeled piece of foil.
5. Using digital calipers, measure the shell height from the hinge to the free edge (anterior-posterior axis) of the shell to the 0.1 millimeter (Figure 1).

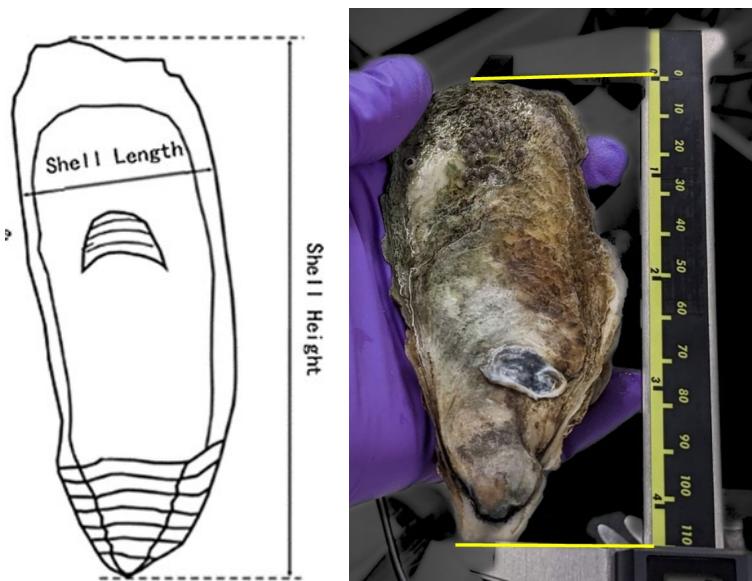


Figure 1. Demonstration of shell measurements (mm) on external anatomy of an oyster shell.

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).

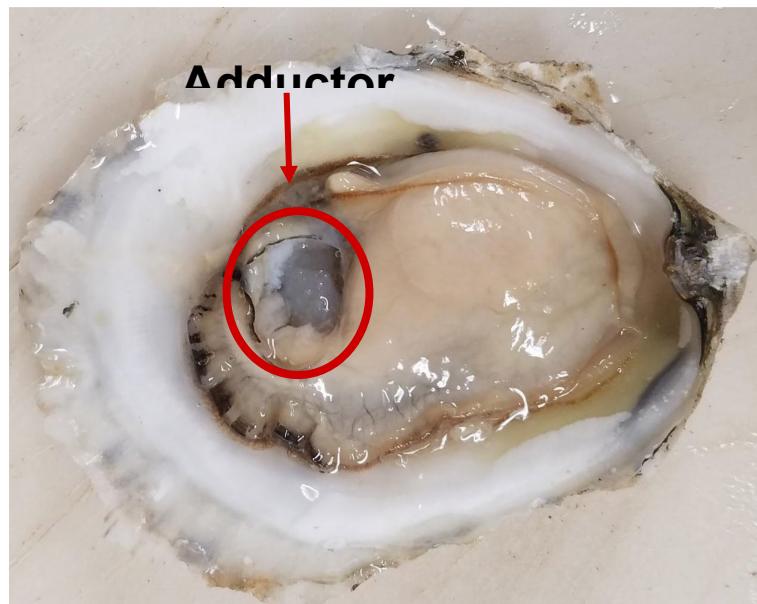


Figure 2. Shucked Oyster

- a. All tools are sterilized between individuals following the steps listed below:
 - i. Freshwater dip
 - ii. Bleach and sand dip to remove tissue (1:10 dilution; 6%)
 - 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.

APPENDIX G

Table 1. Oyster condition rating key (from Quick and Mackin 1971; Histological Techniques for Marine Bivalves Mollusks and Crustaceans. NOAA Technical Memorandum NOS NCCOS 5, 2004.)

Condition Index	Score	Oyster appearance	Photo reference
Very good	1	Animal firm and filling shell cavity; coloration creamy white and evenly textured; usually ready to spawn	
Good	2	Not quite as firm or large as above; usually ready to spawn	
Good minus	3	Coloration less opaque, often slightly yellow or gray	

APPENDIX G

Fair plus	4	Animal distinctly not filling shell cavity; coloration often mottled, with blood vessels and muscle fibers showing through the more translucent epithelium	
Fair	5	Oyster well-developed but not opaque or tending toward white; grayish and translucent; flesh flaccid	
Fair minus	6	Translucency more pronounced	
Poor plus	7	Oyster not well-developed, darker gray, often greenish; pericardial cavity clear; small portion of shell cavity filled	

APPENDIX G

Poor	8	Negative qualities more accentuated	
Very poor	9	Animal distinctly atrophied; coloration dark and uneven, very translucent; seldom more than third of shell cavity occupied; adductor muscle often discolored and transparent even in the normally white sector	

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

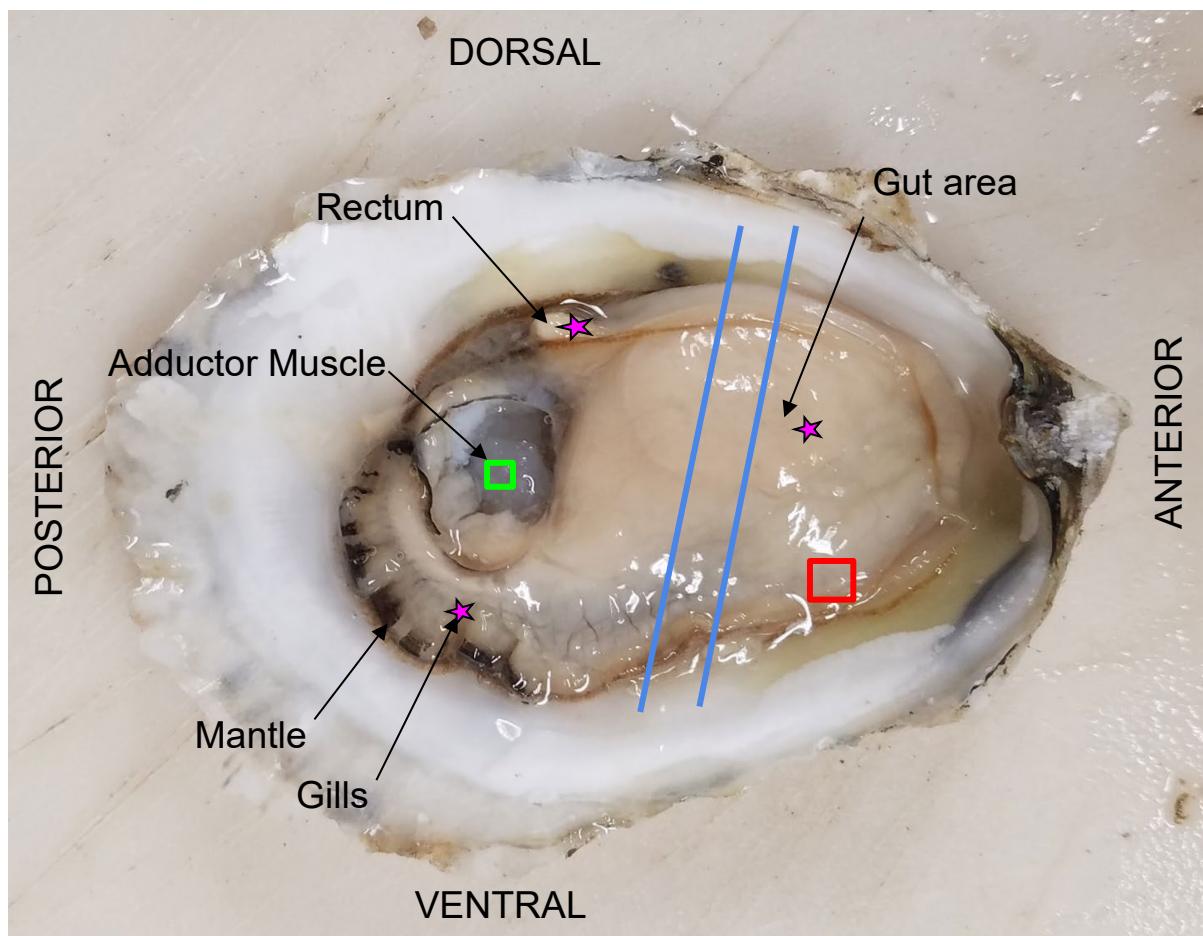


Figure 3: Anatomy of *Crassostrea virginica* for aid in dissection. Blue parallel lines are the location at which histological sectioning should occur. The Red square is the location at which tissue should be extracted for RFTM preservation. Pink stars are the locations at which tissue should be taken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

8. For RTFM:

- a. Using sterile dissecting scissors and forceps, a 5x5mm piece of mantle-edge tissue is excised from just over the palps. Use a ruler as a guide (Figure 3).
- b. Place the tissue in a culture tube containing 5 mL RFTM. 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 5-7 days.
- e. If not analyzed within 7 days of processing, the tube should be placed in a refrigerator in the dark (samples should be analyzed within 2 weeks of being put in the refrigerator).

- f. For tissue analysis, see “RFTM” SOP.
9. For DNA Extractions for triplex qPCR diagnostic assay and tissue archiving:
 - a. Using sterile instruments, duplicate samples of gill, gut and rectal tissues are collected. One set of each tissue is stored together in empty 1.5mL microcentrifuge tubes and held on ice (or cold blocks) until processing is complete. The duplicate set is 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 no more than 0.5mg of tissue is removed (Figure 4). ****A duplicate sample from each oyster will be collected and stored for backup ****
 - b. 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.

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. **All processing steps involving Davidson's fixative should be completed in a fume hood.**
 - 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.
 - c. Using a pathology blade, make a single clean diagonal cut across the body of the animal (Figure 3, Figure 5). Ensure the cross-section includes gill tissue and

avoids the palps. Larger or more elongated oysters may call for a steeply angled diagonal cut to ensure that the proper tissues are being sectioned.



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

- 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.



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

- e. Take the cross section and place it into the cassette (Figure 7), which was prelabeled using pencil.
- f. Each pathology blade should only be used on 1-2 oysters, as needed to ensure sharp,clean cuts.



Figure 7. Cross section placed into histology cassette.

- g. Place the cassettes with tissue into Davidson's fixative (see "*Davidson's Fixative and Histology*" SOP) as samples are being processed. Cassettes should always be fully immersed in solution. Approximately 500mL of Davidson's fixative should be used per sample set of 30 oysters. Clearly label the jar with the date and time samples were fixed as well as sample site information and "in Davidson's fixative". Store in the refrigerator for a minimum of 24 hours (maximum 48 hours).
 - i. After the fixation period is complete and while under a fume hood, drain the fluid into a clearly labeled chemical waste container and fill the jar 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.
- h. For processing and slide preparation, see "*Davidson's Fixative and Histology*" SOP.

11 Clean up

1. All oyster tissue should be thrown in the trash and removed from the laboratory at the end of the day.
2. Oyster shells should be moved to the basement walk-in fridge and stored in a labeled, closed container for donation to shell recycling.
3. All tools, trays, cutting boards, tripours, etc. should be washed with Alconox detergent, rinsed, dried, and put away.
4. All lab surfaces should be sprayed with the appropriate disinfectant and wiped down.
5. Any hazardous chemicals should be returned to the fire safety cabinet.

6. Tissues collected in microcentrifuge tubes for qPCR analysis are stored in the -80°C freezer in Building 1, Room 17. Fresh frozen tissues are placed on racks labeled for short-term processing, while tissues in ethanol are added to storage racks for archived tissue.
 - a. Document addition and location of new samples in the Molecular Lab Inventory Sheet
7. Histology samples preserved in ethanol should be placed in the fire safety cabinet following fixation in Davidson's.
8. All collected samples should be documented in the LISS Oyster Health Project Sample Inventory sheet.
9. Inventory of supplies should be taken to prepare for the next sampling period.
10. All tissue processing activities should be recorded in the laboratory notebook and data sheets should be updated and checked for quality assurance and control.

12 Data Quality Control

Laboratory data will be recorded directly into digital data sheets or recorded 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 physical laboratory data sheets and notebooks will be archived and available at the NOAA Milford Laboratory. Purely digital files (eg. sonde data, photos, digital data sheets, etc.) will be uploaded directly to the NOAA server which is backed up regularly.

13 References

Quick, J. A., and J. G. Mackin (1971) Oyster parasitism by *Labyrinthomyxa marina* in Florida. Fl. Dept. Nat. Resour. Mar. Res. Lab. Prof. Pap. Ser., 13:1-55

END OF SOP

Appendix H – 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 the Long 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 (10g Potassium Iodide and 5g Iodine per 100mL DI H₂O), 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 sterile falcon tubes
- 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
- Microscope Slides, Frosted, 2948-78X25. Corning Inc., Corning, NY.
- Pasteur Pipettes, 13-678-20A. Fisher Scientific, Pittsburgh, PA.

6 Equipment

- Corning Bottle Top Dispenser, 5mL, 6841, Corning Inc., Corning, NY.
- Hot plate with magnetic stirrer, PC-620D, Corning Inc., Corning, NY.
- Vortex mixer, Vortex-Genie 2 Model G-560, 12-812. Fisher Scientific, Pittsburgh, PA.
- 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 extinguished 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. Trash bins containing non-biohazard waste should also be emptied in the facility dumpster in the parking lot. 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 (See Appendix A).
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 and tube racks have been labeled prior to tissue processing.
 - a. Tubes should contain the first initial of the site name (A,F,G,S) and the sample number, 1–30. The tube rack should contain a label with full sample ID details (MMYY SITE_01->30) and tissue processing date.

8. **RFTM (steps 1-3 in procedures) and preparation of culture tubes should be done at least one day prior to sampling.**
9. New electronic data collection sheets should be generated, organized, and dated, and they should be printed or electronically available for data recording. See [TEMPLATE_RFTM](#).
 - a. The template data sheet should be copied and renamed as MMYY SITE_RFTM. This data sheet should be saved in the Google Drive data repository (Project Planning > Data Management > Lab Data > RFTM > Working_Folder).
 - b. Cells B2 and C2 should be updated to reflect the collection date and site for the sample set, and the date the slides are read should be entered in column E (and I if the slide is read by more than 1 person).

10 Procedures

Advance preparation of solutions needed for tissue sampling.

1. Antimicrobial Solution Preparation
 - a. Penicillin-Streptomycin solution (*This solution can be prepared up to 6 months in advance*)
 - i. Autoclave 500mL of deionized (DI) water for 15 minutes at 121°C (15psi) on the slow exhaust setting.
 - ii. After sterile DI water is cool to the touch, add 0.33 g of Streptomycin Sulfate and 0.159 g of Penicillin G. **This amounts to a final concentration of 520 IU/mL Penicillin G and 475 IU/mL Streptomycin sulfate.**
 - iii. Swirl lightly until powder is dissolved.
 - iv. The solution can be stored and maintain potency at 4°C (refrigeration) for 1-2 weeks. For larger batches aliquot desired amount (15mL), label, and store the remaining in the freezer (-20°C) until needed. The solution will maintain potency in the freezer for ~6 months.
 - v. Record the preparation in the lab reagent log and assign a batch number.
 - b. Nystatin Solution (*This solution should be prepared on the same day that RFTM tubes are being prepared*)
 - i. In a small falcon tube mix 4mg of nystatin powder with 4mL of sterile DI water. (0.1g/100mL). This volume is needed to make 70 RFTM culture tubes. Adjust volumes as needed.
 - ii. Mix ingredients well. This solution should be immediately mixed with the Penicillin-Streptomycin solution (10.1.a) and added to the final RFTM tubes (10.2.k).

- c. Combining for final antimicrobial solution (Penicillin-Streptomycin-Nystatin) (*This solution should be prepared on the same day that RFTM tubes are being prepared.*)
 - i. Thaw Penicillin-Streptomycin to room temperature in a warm water bath. 40mL of penicillin-streptomycin and 4mL nystatin solution (see 10.1.b) is needed to make 70 RFTM culture tubes. Adjust volumes as needed.
 - ii. Combine penicillin-streptomycin and nystatin solutions in a 50mL sterile tube. Cap the tube and invert several times to mix. Keep aside until ready to combine with RFTM (See 10.2.k)
2. Ray's (1952) Thioglycollate Medium (RFTM) preparation
- a. In a flask, add Sodium chloride (NaCl) and dehydrated Fluid Thioglycollate medium (FTM) into distilled water. Adjust quantity of ingredients based on the desired number of tubes. Suggested amounts:
- | Final volume | # of culture tubes | NaCl (g) | FTM (g) | Distilled water (mL) |
|--------------|--------------------|----------|---------|----------------------|
| 1L | 200 | 22 | 29.3 | 1000 |
| 650mL | 130 | 14.3 | 19 | 650 |
| 350mL | 70 | 7.7 | 10.3 | 350 |
- b. Preheat the heat/stir plate at the highest setting (varies by heat plate), put the flask on a heat plate, 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. This can take 30-40 minutes depending on the heat plate. Keep a close eye on the flask. It will start from a gray/blue to pink color. Once it turns to pink, turn the heat down slightly to avoid overflow.
 - d. While the solution is heating, prepare a water bath at room temperature. Obtain clamps/tongs and heat safe gloves to transport the flask.



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

- e. The solution will begin to change to a golden-yellow color (Figure 1). When this color is achieved immediately take the mixture off of the heat plate. **Do not let it overflow from boiling.**
- f. Using tongs or heat gloves, carefully place the flask into a tub of room temperature water to cool down. Note that as the solution cools it will turn back pink.
- g. After the flask is cooled to the touch, dispense 5mL of solution into each 15mL culture tube using the "Corning Bottle Top Dispenser".
 - i. If needed, obtain the instruction Manual.
 - ii. Attach the telescopic tube (adjustable) to the dispenser. Be sure that the tube touches the bottom of the flask. This may take a few length adjustments of the telescopic tube.
 - iii. Make sure the reflux tube (smaller tube) is attached to the dispense.
 - iv. Screw the dispenser onto the desired bottle.
 - v. To purge air from the dispenser, turn the discharge tube (outer spout) to 90 degrees. Set a small volume (1-2 mL). Raise and press the dispenser a few times until liquid exits the reflux tube.
 - vi. Return the discharge tube to 0 degrees or the position to allow liquid to dispense.
 - vii. To dispense, adjust to the desired volume (5mL). Raise the housing until it stops and press the housing to the lowest point. The movement should be slow and consistent to avoid air bubbles and achieve the exact amount desired.
- h. Screw the caps loosely onto the tubes.
 - i. The tubes with medium are then autoclaved for 15 minutes at 121°C (15psi) on the slow exhaust setting. When autoclaving is finished, allow tubes to cool to the touch.

- j. Pipette 550 μ L of antimicrobial solution (prepared in 10.1.c) to each RFTM culture tube. As the solution is added to each tube, cap tightly and vortex quickly for 1-2 seconds. Continue until solution is added to all tubes.
- k. Label the culture tubes and tube racks according to the instructions in 9.7.
- l. Record the preparation in the lab reagent log and assign a batch number. Log reagents used to prepare original RFTM media, nystatin solution, and the penicillin-streptomycin solution batch number.
- m. Place the culture tubes in the refrigerator and in the dark until ready for use.
The RFTM may change color from yellow to pink when vortexed. They should return to the yellow once cooled in the refrigerator
 - 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.
- n. ***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°C) until the pink disappears. Cool to <45°C before inoculating with tissue.

3. Lugol's Iodine Solution for staining

- a. Stock product being used is Lugol's Iodine containing 10g Potassium Iodide and 5g Iodine per 10mL of DI water.
- b. Calculations for working solution:
 - i. 10mL stock Lugol's + 20mL DI water = 3.33g Potassium iodide and 1.66g Iodine per 100mL

WOAH recommends: 2g Potassium Iodide and 1.3g Iodine per 100mL

4. Inoculation and Incubation of oyster tissues in culture tubes

- a. Using sterile dissecting scissors and forceps, a 5x5mm piece of mantle-edge tissue is excised from just over the palps. (Figure 2) **Full protocol for tissue excision can be found in the Oyster Tissue Processing SOP**
- b. Place the tissue in a culture tube containing 5 mL (500 μ L) RFTM, 0.5mL Penicillin-Streptomycin and 50 μ L nystatin solutions (See 10.1.k). Use a sterile probe to fully submerge the tissue in the bottom third of the RFTM.

- c. Screw cap back on tightly.
- d. Incubate culture tubes with tissues in the dark at room temperature and for at least 5 days.
- e. If not analyzed within 7 days of inoculation, the tube should be placed in a refrigerator in the dark. Tissues should be analyzed within 2 weeks after being placed in the refrigerator.

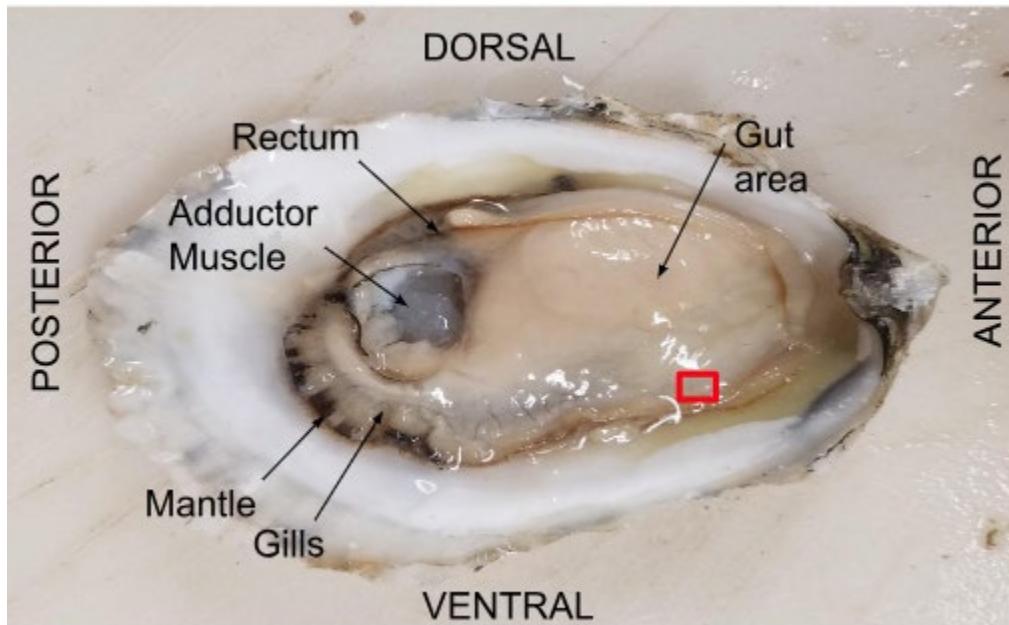


Figure 2: Anatomy of *Crassostrea virginica* for aid in dissection. The Red square is the location at which tissue should be extracted for RFTM preservation.

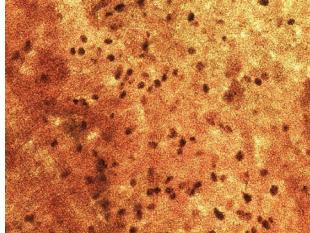
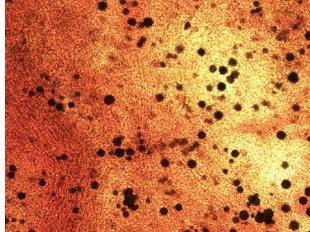
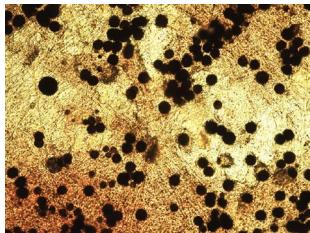
5. Tissue Analysis/Diagnostics

- 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, or 100x as required 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 5-300 μ m. 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).

APPENDIX H

- i. Each month, 10% of all samples should be independently read by two people for quality control.

Table 1: Mackin Scale (Ray 1954a, 1954b). 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%	

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

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

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

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

- g. See [Triplex qPCR protocol](#) for molecular approach to quantification of *P. marinus* DNA in oyster tissue.

6. Results reporting

- a. Results will be recorded in the lab notebook and will be transcribed into the digital data sheet (see 9.9).

7. 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 30 minutes at 121°C (15psi) on the slow exhaust setting.

APPENDIX H

Once the liquid is cool to the touch, it can be flushed down a sink that discharges to the sewage treatment line.

- c. The Corning Bottle Top Dispenser:
 - i. Turn the discharge tube to 180 degrees to allow the remaining to flow back into the bottle.
 - ii. Screw off the dispenser from the bottle and drain the telescopic tube by purging as described above.
 - iii. Fill a bottle with distilled water or alcohol and turn the discharge tube to 0 degrees or dispense mode.
 - iv. Dispense multiple times until the dispenser is clean and cleared of any reagent.
 - v. If necessary, disassemble the dispenser and clean the components.
 - vi. The dispenser is autoclavable to sterilize. Remove the reflux tube and telescopic tube . Steam- sterilize dispenser at 121°C for 15 minutes.
- d. Dissection tools used for processing tissue should be sterilized using an ethanol flame followed by washing with soap and water. Additional supplies such as trays, beakers, etc. should be washed with soap and water.

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 (See 9.9 and 10.9 for specific instructions). 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 physical laboratory data sheets will be archived and available at the NOAA Milford Laboratory. Purely digital files (eg. sonde data, photos, digital data sheets, etc.) will be uploaded directly to the NOAA server which is backed up regularly.

12 References

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.

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 (1:10 dilution)
 - iii. Freshwater dip
 - iv. 95% ethanol dip
 - v. Ethanol is burned off using a flame (allow to cool before reusing)
- b. [RFTM datasheet template](#)
- c. Oyster Tissue Processing SOP
- d. [Triplex qPCR protocol](#)

END OF SOP

Appendix I – 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°C freezer
- -80°C 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 occurring.

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.

APPENDIX I

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. 4M Urea buffer (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°C (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.

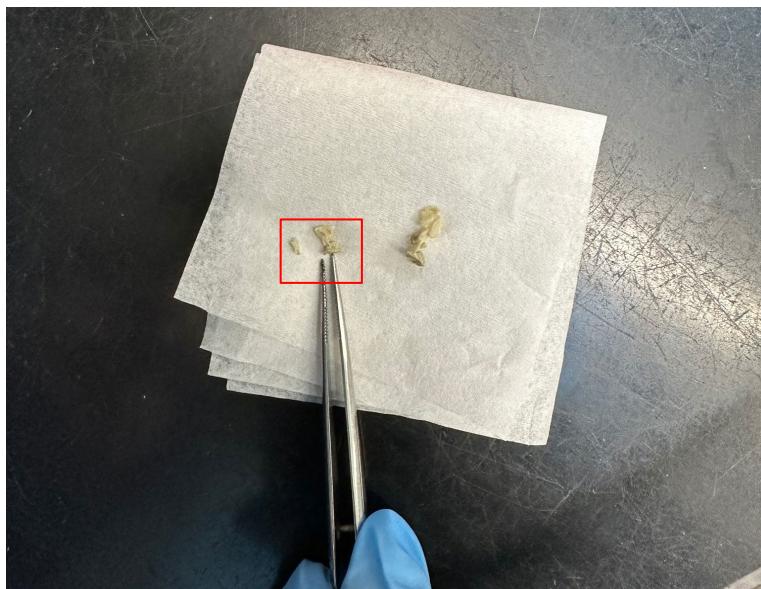


Figure 1. Size comparison of 0.5mg of gut, mantle, and gill tissue samples from eastern oysters for DNA extraction. Size of each tissue type is no larger than the tip of a fine-point forceps.

- 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 180 μ L 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 μ L of 100TE buffer (to adjust the pH to 8.0)
 - ii. 50 μ L of ammonium acetate
 - iii. 400 μ L 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/ μ L 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/3 μ L in 50 μ L aliquots.

Calculate using the equation: $C_1V_1 = C_2V_2$ where,

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

V_1 = Volume of neatstock (Unknown),

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

V_2 = Total volume of analyte (50 μ L)

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

The required volume (μ L) 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.
 - I. 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

APPENDIX I

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 J – 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: ACAGGTCAGTGATGCCCTAG
MSX/SSO Reverse Primer: TSGRGATTACCYSGCCTTC
MSX Probe: SHEX/ TTGCACGCAACGAGTTAACCTTGCCTG /3BHQ_1
 - SSO Probe: 5Cy5/AATGACCCAGTCAGCGGGCCGA/3BHQ_1
- Plasmid standard Curve (10 to 10⁸ 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 R² 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.

APPENDIX J

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
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°C or -80°C). 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°C 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°C for 60seconds
 - b. 40 cycles of: 95°C for 15 seconds and 60°C for 30 seconds
 - c. 4°C 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°C
 - 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 (10⁸)
 - 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 toolbar.
 - 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 R² values. Ideally an efficiency of 95+% and an R² 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 $\leq 2.0\%$ 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 $\leq 2\% C(t)$) 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 1 μ l 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 K – 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 - Delonized 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°C (standard refrigeration).
 - f. Post-refrigeration, dispose of the fixative into a designated chemical waste container under the fume hood using the designated Davidson's Fixative funnel.
 - g. Add 70% ethanol to cover cassettes and store at 4°C (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 $\frac{1}{4}$ 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 $\sim 42^{\circ}\text{C}$. (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 $\frac{1}{8}$ 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 Eosin₂ (HHE₂) (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	NaHCO ₂ (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

- 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.
- Place one drop of mounting fluid on the tissue on the slide.
- 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°C for 2-5 days to allow the mounting medium to harden.
 - f. After hardening of the medium, clean, touch up labels and file slides. Remove 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 L – 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)

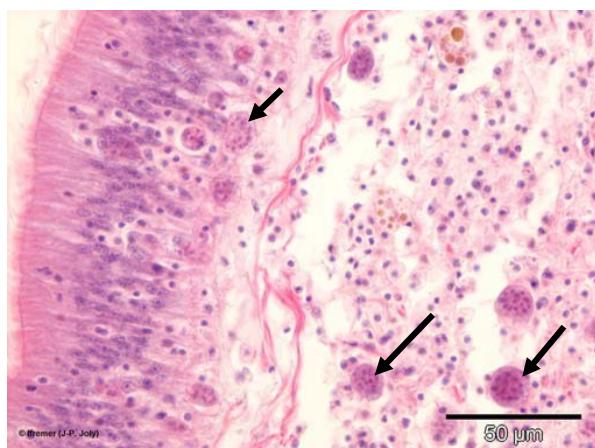


Figure 1 : Plasmodia of *Haplosporidium nelsoni* in connective tissue and intestine epithelium of *Crassostrea virginica*, H&E staining. Image from EURL Diagnosis by histopathology of *Haplosporidium* sp. in oysters.

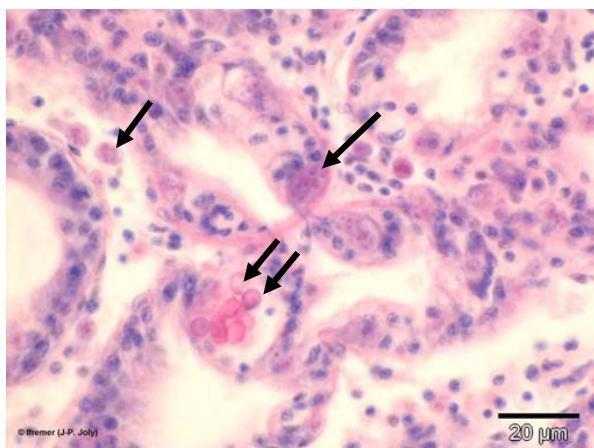


Figure 2 . Plasmodia (arrows) and spores (double arrow) of *Haplosporidium nelsoni* in connective tissue and digestive diverticula epithelium of *Crassostrea virginica*. H&E staining. Image from EURL Diagnosis by histopathology of *Haplosporidium* sp. in oysters.

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)

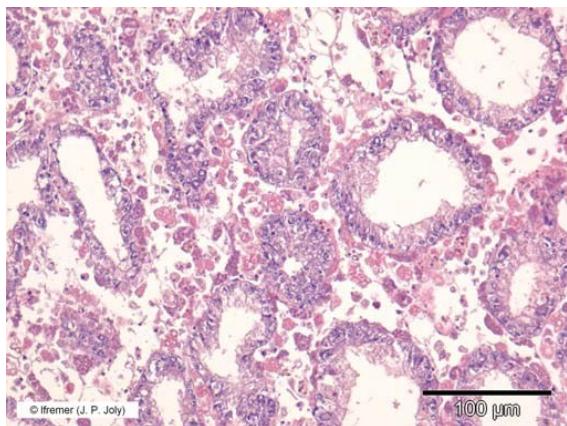


Figure 3 . Digestive gland of a *Crassostrea virginica* heavily infected by *Haplosporidium costale* with many plasmodia and sporocysts in the partially destructed connective tissue, H&E staining. Image from EURL Diagnosis by histopathology of *Haplosporidium sp.* in oysters.

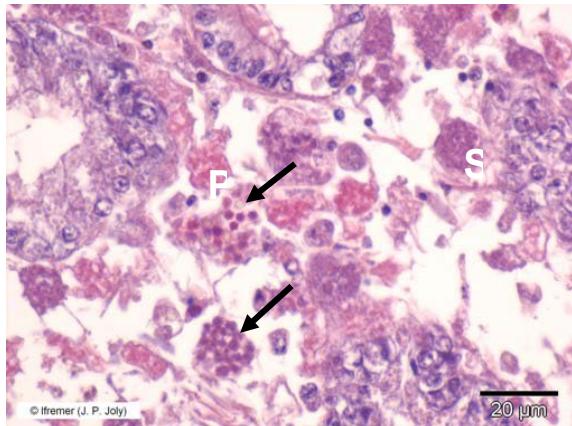


Figure 4 . Higher magnification of figure 3. Note the plasmodia (P), the sporocysts (S) and the spores (arrows) in the connective tissue, H&E staining. Image from EURL Diagnosis by histopathology of *Haplosporidium sp.* in oysters.

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 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).

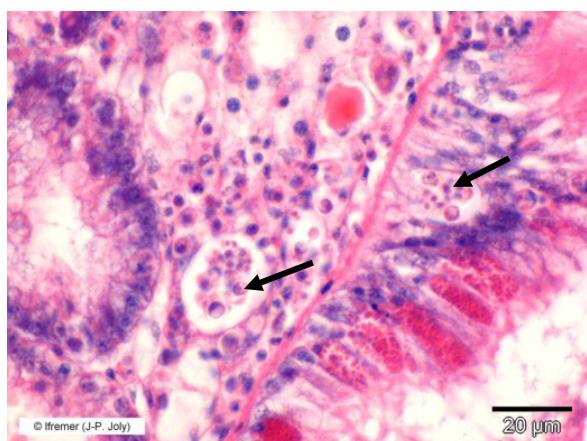


Figure 5. *Perkinsus marinus* cells in the connective tissue of the digestive gland and in the gut epithelium of an infected *Crassostrea virginica* oyster, H&E staining. Image from Ifremer Diagnosis by histopathology of *Perkinsus* sp. in molluscs, European Union Reference Laboratory.

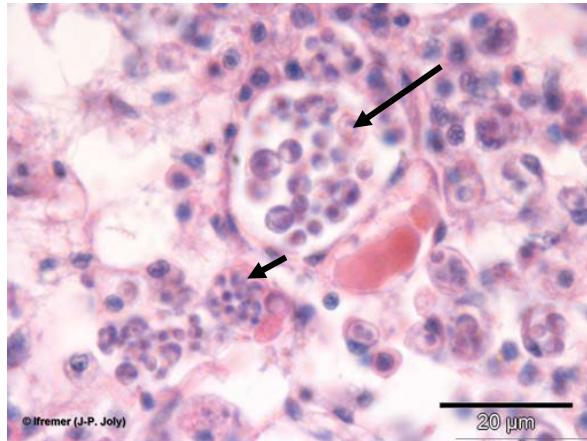


Figure 6. Different stages of *Perkinsus marinus* trophozoites in the connective tissue of the digestive gland (*Crassostrea virginica*), H&E staining. Image from Ifremer Diagnosis by histopathology of *Perkinsus* sp. in molluscs, European Union Reference Laboratory.

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 (Heavy)	4	Systemic infections with cells very abundant in connective tissues of all organs

4. Results reporting

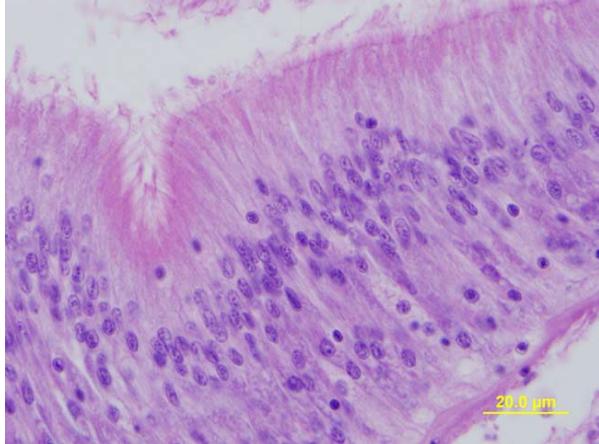
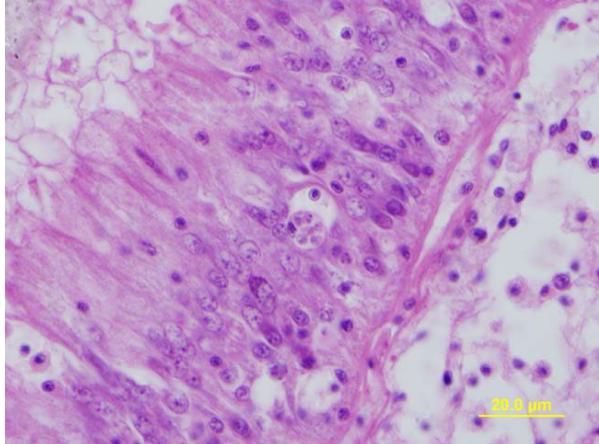
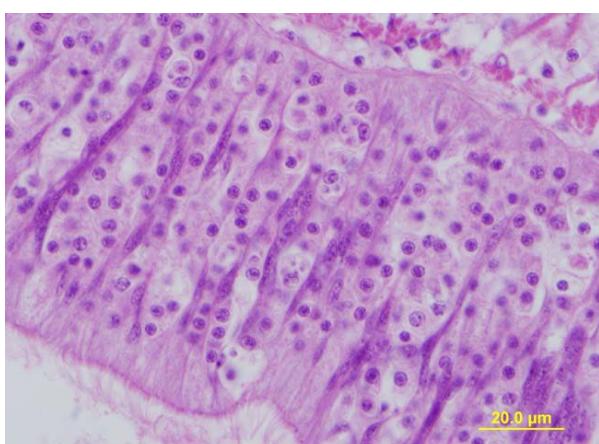
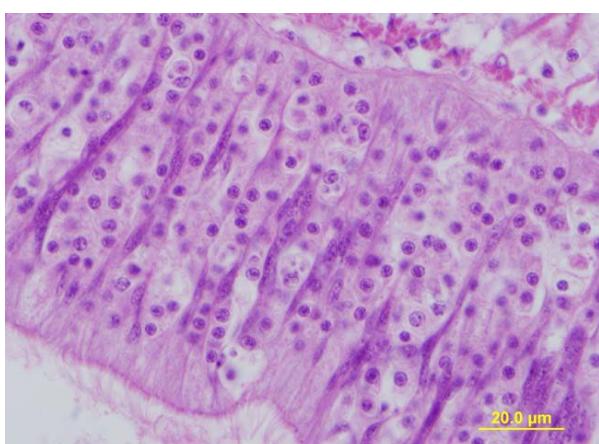
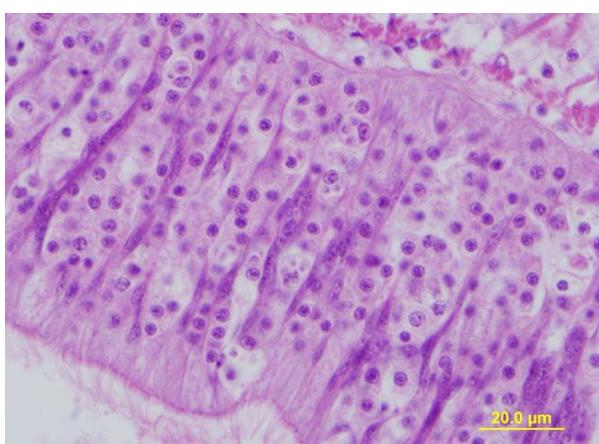
- a. After reviewing all slides, calculate the population prevalence and intensity level of *Perkinsus marinus* and *Haplosporidium spp.* infections in a given sample using the following calculations:

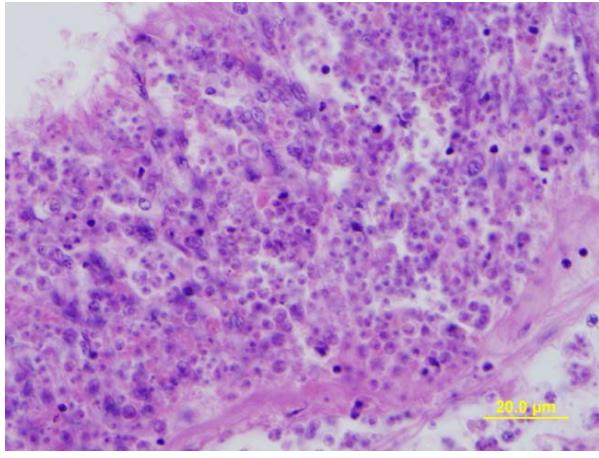
$$\text{i. } \textit{Prevalence (\%)} = \frac{\textit{\# of infected individuals per sample}}{\textit{total \# of individuals collected per sample}}$$

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

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

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	 A light micrograph showing a dense population of small, dark-staining parasites (P. marinus) within the spaces of connective tissue. The parasites are numerous and appear to be colonizing the interstitium. A scale bar in the bottom right corner indicates 20.0 micrometers.
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 M – 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 [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.

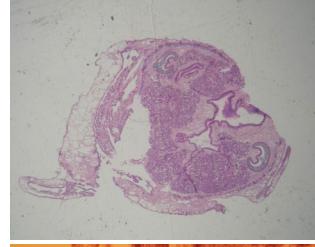
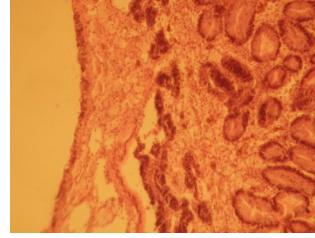
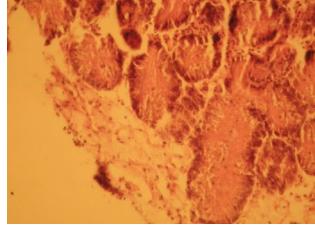
6. All data collection sheets should be organized, dated, and printed or electronically available for data recording.

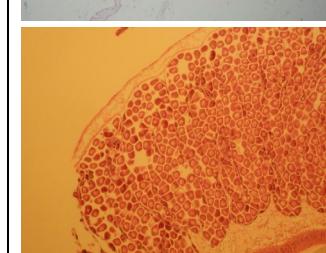
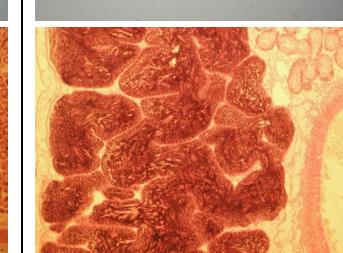
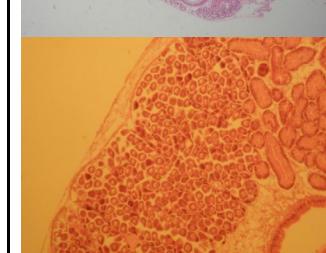
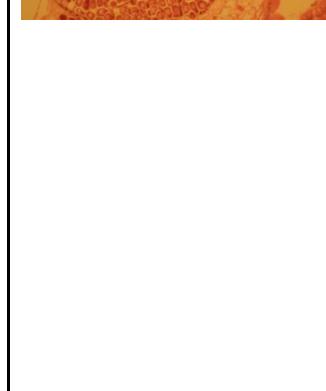
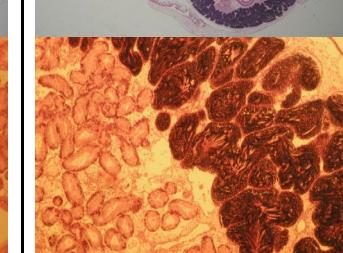
8 Procedures

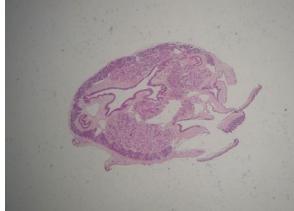
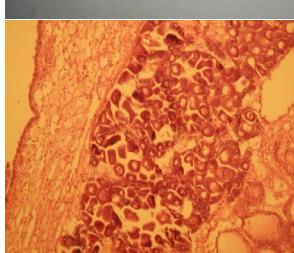
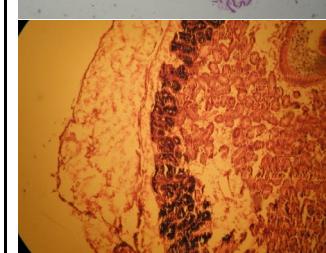
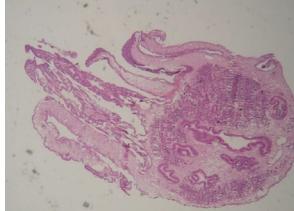
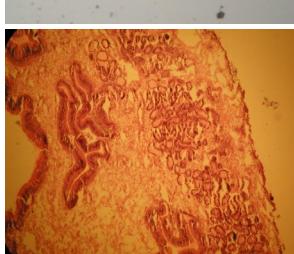
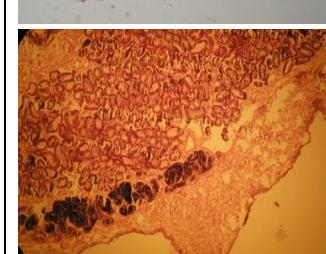
1. Reproductive conditioning

- 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/Indeterminate	Follicles are nonexistent or elongated, with walls consisting of undifferentiated germinal epithelium. Sex cannot be determined		
1	Developing	Gametogenesis initiated, but cannot tell the sex, can only see the follicles.	   	

2	Early active	<p>Follicles contain oogonia or spermatogonia and primary oocytes or spermatocytes predominate in the follicles, some spermatazoa may be apparent. Sex is distinguishable.</p>	  	 
3	Late / Mature	<p>Clearly identifiable gametes (ova or spermatozoa) in the follicles; presence of ova with distinct nucleus and nucleolus, spermatozoa oriented with tails toward the follicle lumen. This can range from partially full to full, the amount of gonad full is less important than the developmental stage of the actual gametes.</p>	  	 

4	Spawning	<p>Gametes are in the gonadal ducts - but there are still significant gametes present.</p>	 	 
5	Post spawning	<p>Spawned follicles have gaps devoid of gametes; although numerous gametes may still remain, follicle walls may be broken.</p>	 	 
6	Reabsorbing	<p>Follicles have a shrunken appearance and contain numerous phagocytes and products of reabsorption; gametes, if present, are few and refractory, and development is not evident.</p>		

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.

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.

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). <https://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.

END OF SOP

Appendix N – Monthly Sonde Maintenance SOP

1 Purpose

The purpose of this SOP is to provide concise guidance and methodology for maintaining the Aqua Troll 600 data sonde(s) on a monthly basis to accurately collect temperature, salinity, pH, dissolved oxygen (DO), and chlorophyll-a data to characterize site water quality.

2 Scope

This SOP is pertaining to the EPA Long Island Sound Study funded Oyster Health project that is incorporating water quality metrics to understand host-pathogen- environment relationships.

3 Definitions/Acronyms

4 Safety Precautions

All team members will wear appropriate clothing dependent on weather conditions including but not limited to waders, rubber boots or protective footwear, gloves, hats, sunglasses, long sleeve shirts, and pants. Thick protective gloves (e.g garden gloves) should be worn when handling fouling organisms. Team members will wash hands thoroughly after field trips end. A first aid kit will be present for any injury. Extra water will be provided to avoid dehydration or heat stroke. Team members will take regular breaks when needed.

Exercise weather-appropriate field safety measures by monitoring conditions before and during the trip. Do not perform fieldwork during dangerous conditions (e.g. lightning, extreme winds, extreme floods). Do not visit field sites alone (use buddy system). Inform PIs of dates and times of fieldwork. Confirm safe return to the lab. At intertidal sites, perform procedures during low tide. At subtidal sites, divers are to follow NOAA diving regulations according to the instructions of the lab diving coordinator (Barry Smith, barry.smith@noaa.gov).

5 Reagents/Media

1. Bottle of distilled water (1 L) for rinsing sonde and probes.
2. In-Situ [pH filling solution](#) for replacing fluid inside pH sensor (SKU 0056900). Stored in the Garage with LISS field supplies.
3. In-Situ [Quick Cal solution](#) for calibrating pH sensor (SKU 0033250). Stored in the fridge in Building 1 Rm 29. Refer to note under Section 8 (Quality Control).

6 Supplies/Equipment

1. Field Tablet (containing VuSitu app).
2. Robust zip-ties (e.g., 24 inch) for securing the sonde to the PVC housing at subtidal sites.
3. $\frac{15}{16}$ inch socket wrench
4. $\frac{15}{16}$ inch open-end wrench

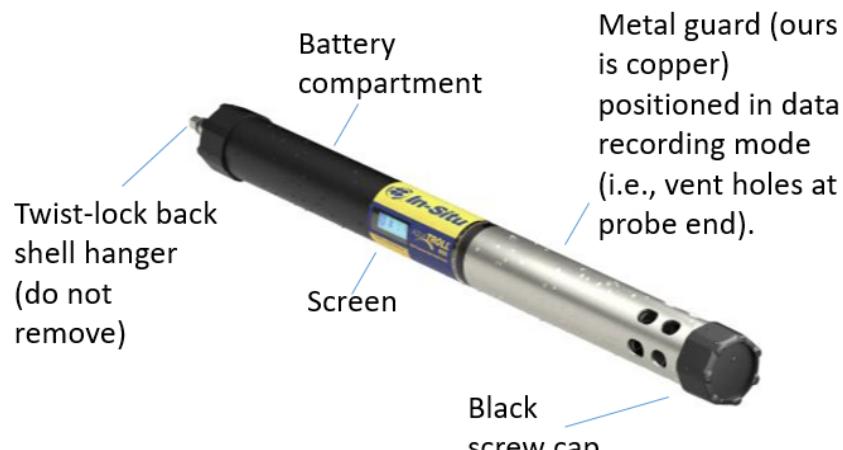
5. Adjustable wrench
6. Sonde supply box
 - a. High vacuum grease
 - b. RDO sensor Calibration sponges
 - c. Extra pins and bolts for housing
 - d. Desiccant tube
 - e. Screw drivers
 - f. Hex driver
 - g. Allen key
 - h. Sonde wipers (Aqua Troll part #0078940)
 - i. Alcohol pads
 - j. D batteries (minimum of 2, Energizer or Duracell only).
 - k. Paper towels (for collecting discarded pH filling solution).
 - l. Q-tips or cotton buds
7. Bottle with fresh water for cleaning sonde
8. Towel / cloth for wiping things down.
9. Dish scrubbing pad
10. Scrub brush
11. Shucking knife
12. 500 mL empty container with sealable top
13. First aid kit
14. Hand sanitizer
15. Wire cutters
16. Gray floating tray

7 Quality Control

All team members will be trained to complete all field tasks, including training on data entry requirements for each specific task. Environmental monitoring data will be regularly checked for drift and sensors will be calibrated when necessary.

8 Preparation (At the lab)

1. Restock the servicing tools and supplies in the gray floating tray, specifically in the servicing kit (clear container with black snap locks, Appendix A).
2. Ensure that all SOPs and field notebooks are available offline on the field tablet.
 - a. Monthly Sonde Maintenance (this SOP)
 - b. Monthly HOBO Maintenance
 - c. Water Sampling and Chemistry
 - d. Sonde Sensor Calibration
 - e. Sonde Field Notebook
 - f. Site specific field notebook
3. Ensure that the field tablet is fully charged.



In-Situ Aqua TROLL 600 Multiparameter Sonde

Figure 1: Anatomy of the Aqua Troll 600.

9 Procedures

1. Removing sonde from housing
 - a. Intertidal sites
 - i. Obtain the gray tray that has all appropriate tools for servicing the data sonde (see Appendix A).
 - ii. Attach the gray tray to self using the string and clip so that the tray does not drift away with the current. (Figure 2)
 - iii. Wade to data sonde housing with the tray.
 - iv. Using a socket wrench and pliers, unscrew the stainless steel security bolt that passes through the exposed top end of the PVC housing.
 - v. ***Place the screw and bolt in the gray tray immediately so you don't lose it.***
 - vi. Unscrew and remove the PVC cap using the tongue and groove pliers if necessary.
 - vii. The cap is attached to a clear tether (fishing line) that is linked to the sonde. Pull the tether to lift the sonde out of the PVC housing. Place the sonde and cap into the gray tray.
 - viii. Servicing can be done directly at the sonde or by returning to shore depending on time, tide height, and weather conditions. (Figure 3)



Figure 2. Gray tray tethered to self.

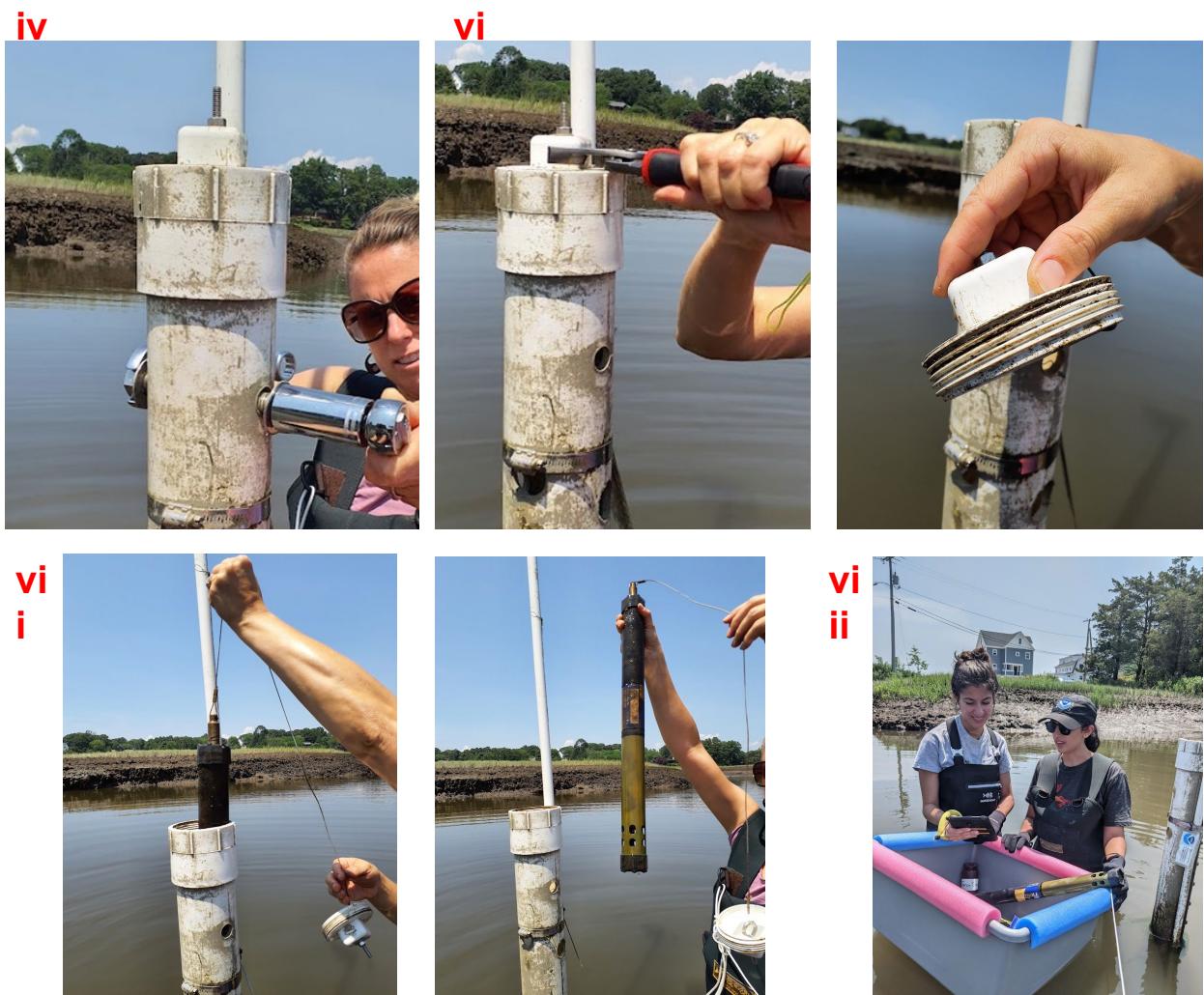


Figure 3. Demonstration of removing sonde at intertidal sites from PVC housing. Red roman numerals are following the specific steps in the text above.

b. Subtidal sites

APPENDIX N

- i. *Laurel Hollow*: The data sonde is attached to the mushroom anchor (marked by a NOAA buoy). Divers will need to:
 1. Unclip the parachute cord that loops from the top of the data sonde around the shank of the anchor.
 2. Use wire cutters to cut a zip-tie that secures the data sonde to the PVC housing (the one that passes through the vent holes of the PVC housing, not the zip-ties holding the PVC housing to the anchor itself).
 3. Remove the data sonde from the PVC housing (it also has a HOBO data logger attached near its top end).
 4. Return the sonde + HOBO to the support boat or shore party.
- ii. *Gold Star Beach*:
 1. **Access Cornell Extension's FLUPSY (first left on the town dock; gate combination = 2+4 together, then 3).**
 2. The data sonde is deployed on a 25 pound mushroom anchor from inside a locked hatch on the floating dock.
 3. Obtain the key to the floating dock. Demetrios Caroussos has the key (if there, he will open it; otherwise, make arrangements for him to leave the key in advance: email dc2233@cornell.edu, cell 631-418-6570).
 4. Pull up the 25 pound mushroom anchor from the seabed and place it on the dock.
 5. Unclip the line that attaches the top end of the sonde to the anchor.
 6. Cut the zip-tie that holds the sonde inside the PVC housing (not the ones attaching the PVC housing to the anchor).
 7. Remove the data sonde from the PVC housing.



Figure 4: Map showing approximate location of all gear deployments at Gold Star. Inset shows an image of the sonde housing secured to the mushroom anchor.

2. Remove any accumulated fouling organisms from the exterior of the sonde before proceeding. Use the dish scrubbing sponge or bristle brush. Barnacles may need to be removed with a shucking knife.
3. Downloading data
 - a. Log into the Samsung tablet .
 - b. Invert the data sonde so the sensors are facing upwards (the blue screen of the data sonde should illuminate and initiate bluetooth connectivity). (Figure 4). Once connected, the sonde can be back in the upright position to ensure no damage to probes.
 - c. Open the VuSitu app and click the sonde it detects (each sonde has a unique serial number which is logged in the downloaded data folder, in addition to any location information provided during setup).
 - d. Once connected, click *Logging*.
 - e. On the next screen, click *Download*. Choose the option to download ‘only new data’.
 - f. Downloading takes a few minutes (~5-10 min). During this time, clean the probes and area around them of any fouling (see section 4 ‘Cleaning probes’)
 - g. Once the data has been downloaded, a screen of the last 5 readings will appear. Take note of the most recent readings for temperature, salinity and pH in the field notebook (needed for discrete water chemistry samples). ***Pay attention to the values in the event that drift occurs. Sensors may need calibration:***
 - i. The pH sensor should be calibrated every 10-12 weeks or if drift occurs. See Sonde Calibration SOP for specific instructions.
 - ii. RDO sensors should be calibrated every 12 months. See Sonde Calibration SOP for specific instructions.
 - iii. Conductivity, Temperature and Chlorophyll-a sensors should be calibrated every 12 months. (this is factory calibration that requires sending the sondes in for servicing)
 - h. Then, Click *Save As*. Save the data to the shared drive (shortcut to *Data_Sondes* folder; full path: *Project Planning / Data Management / Field Data / Data_Sondes / Sonde Data / Site specific folder*).
 - i. Rename file as MMYY SITE_Sonde where MMYY corresponds to the month and year of the download date.
 - j. Once completed, return to the home screen (back out of the *Logging* screen using the top left arrow).
 - j. The homescreen should indicate that the logging session is still in progress (logging is paused during data download, but it restarts automatically once the download procedure has been completed).
 - k. If you’ve finished working with the sonde, click *Disconnect*.

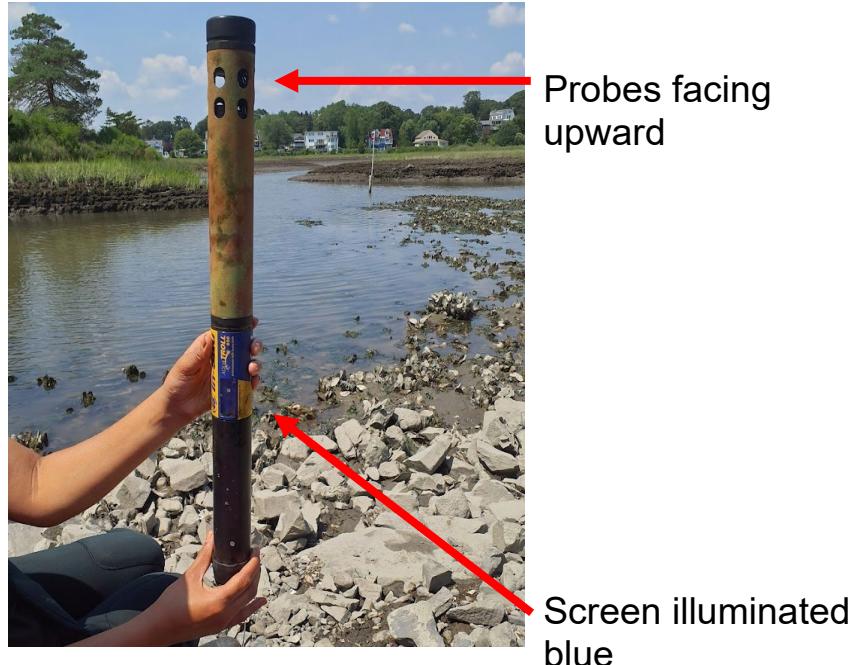


Figure 5. Inversion of the Sonde to initiate bluetooth connection.

4. Cleaning probes

- a. Remove the copper guard from the sonde by twisting counterclockwise. Gently lift over the probes avoiding disturbing the wiper. (Figure 6)



Figure 6. Copper guard removed from sonde to expose sensors.

- b. Place the copper guard and sonde in the gray float tray.
- c. Assess the biofouling on the wiper. If the fouling is significantly covering the wiper and is difficult to remove, replace the wiper with a new one.
 - i. Using the thin screwdriver (in the sonde supply box), unscrew the wiper from the wiper poll. The screw is very small and is in the bottom corner adjacent to the wiper poll. If fouling is covering the screw, **gently** remove fouling from that area using a shucking knife. ***Be sure to never touch the probe sensors with the shucking knife to avoid damage.***

- ii. Gently lift the wiper up and off of the wiper poll and discard into the gray trey.
- iii. Obtain new wiper and slide into place. Secure by screwing the small screw back into place.
- d. Using Q-tips gently remove fouling from the sensors. Rinse periodically with water as needed.
 - i. *The pH sensor should not be directly touched using the Q-tips. Only clean the area around the sensor.*
 - ii. Conductivity, Temperature, Chlorophyll-a, and RDO can be **gently** wiped using the Q-tip and water. (Figure 7)

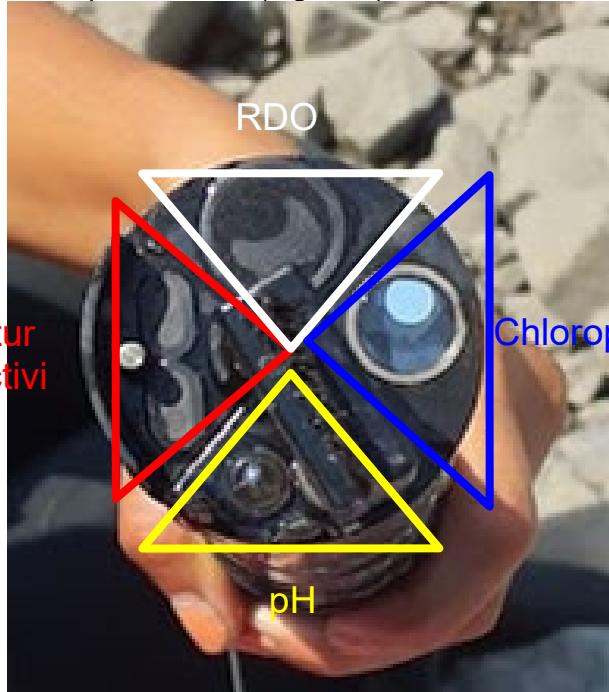


Figure 7: Identification of sensors on the sonde.

- e. If there are calcified organisms in crevices, use the shucking knife or Hex screwdriver to remove. ***Do not use these tools to remove calcified animals directly on sensor screens. (see Appendix B for removing calcified materials)***
 - i. In the event that calcified organisms are present directly on the sensors, decalcification will need to occur in the lab. Follow the instructions in the Sonde Deployment SOP to store the sonde for transport back to the lab. If a backup sonde is available in the field kit, follow the Sonde Deployment SOP instructions to set-up and deploy the replacement sonde.
 - f. Once sensors are clean, remove fouling from the inside of the copper guard and gently twist back onto the sonde clockwise in the data logging position (holes near probes/sensors).
- 5. Battery Change (Figure 8)
 - a. *If the sonde battery level is less than 50%, change the batteries. Duracell or Energizer name brands preferred (recommended by InSitu).*

- b. First ensure that the data has been downloaded from the current logging session (as described above in section 9.3 Downloading data). Stay connected to the VuSitu app.
- c. End the current logging session (click *Logging*, then *More*, then *Stop*).
- d. Return to the app main screen, then click *Disconnect* to disconnect the sonde from the tablet.
- e. Dry the sonde with a towel or cloth. Unscrew the battery compartment (black part of the sonde). It unscrews at the joint between the middle screen section of the sonde and the black top section.
- f. Remove the two installed D batteries and replace them with two new ones. Put the old batteries in the dry box with servicing tools. New batteries are in a ziplock bag. Keep old batteries and new batteries separated.
- g. Check the small desiccant capsule inside the battery compartment and replace it if pink, as follows:
 - i. Use an Allen wrench to remove and check the desiccant compartment/color (Figure 9).
 - ii. If the present desiccant is pink, that is the indicator that it needs to be replaced.
 - iii. Snap a new desiccant pill into the compartment if color indicates replacement is needed.
- h. Clean and dry the O-rings of the battery compartment.
- i. Apply a thin layer of vacuum grease over the surface of the O-rings (tubes of grease are in the red box).
- j. Screw the sonde back together to seal the battery compartment. *There will be a distinctive click when it is screwed on fully.* If the sonde's blue screen displays any prompts, complete those steps.
- k. ***Ensure that a logging session has been activated (shown in app home screen before disconnecting) before redeploying the sonde.***



Open the battery compartment.

Install alkaline batteries.

Use Allen wrench to remove and check desiccant color. If pink, replace.

Close battery compartment. LCD screen should activate.

Figure 8: changing the batteries in the data sonde



Figure 9: Demonstration of removing desiccant pill.

6. If drift is apparent or regularly scheduled calibration is needed, see Sonde Sensor Calibration SOP and complete calibrations at this step.
7. Redeploying Sonde
 - a. Before deploying the sonde, make sure that the copper guard (covering the probes) is attached in the data logging position and that the sonde has been set to record data. The copper guard vent holes should be near the sensors to allow water flow.
 - b. Intertidal sites
 - i. Using a cleaning brush scrub or shucking knife, clean the PVC housing and PVC cap to remove any mud, algae or fouling organisms.
 - ii. Insert the sonde into the PVC housing and gently lower it with the probes facing downward until it hits the cross bolt threaded near the bottom of the housing.
 - iii. Screw the PVC cap (still tethered to the data sonde) into the top of the PVC housing. Do not over tighten.
 - iv. Insert the stainless steel security bolt through the top of the PVC housing (i.e., through the two holes drilled near the top of the housing), then screw on its corresponding nut until it's snug.
 - c. Subtidal sites
 - i. Laurel Hollow
 1. Take the sonde and its attached HOBO and tether/clip + 1 large zip-tie + the brush to the mushroom anchor (by boat, or swim from shore).
 2. Dive to the mushroom anchor and use the brush to clean the inside and outside of the PVC housing, making sure the vent holes are clear to allow water flow.
 3. Place the sonde inside the PVC housing with the probes facing downwards.
 4. Loop the parachute cord (attached to the top of the sonde) around the anchor shank and clip it back on itself.
 5. Pass a zip-tie through one of PVC vent holes and out another vent hole (both about half way up the sonde) and use it to secure the sonde tight against the inside of the PVC housing.

ii. *At Gold Star Beach:*

1. Use the brush and shucking knife to clean the inside and outside of the PVC housing, making sure the vent holes are clear to allow water flow.
 2. Check the hose clamps and zip ties securing the housing to the anchor and reinforce as needed.
 3. Place the sonde inside the PVC housing with the probes facing downwards.
 4. Clip the tether from the anchor onto the top of the data sonde.
 5. Pass a zip-tie through one of the PVC vent holes and out another vent hole (both about half way up the sonde) and use it to secure the sonde tight against the inside of the PVC housing.
 6. Lower the mushroom anchor to the seabed and fasten it on a cleat.
 7. Close and lock the trap door of the FLUSPY.
8. Return to lab & clean up
 - a. Rinse all tools (wrenches, pliers, wire cutters) with fresh water, dry with a towel, and spray with WD40 (kept on field gear shelves, extra in Rm 29).
 - b. Connect the Samsung tablet to WiFi in order to sync the shared Google drive (contains the downloaded data files from the sonde and HOBO).
 - c. Used batteries can be disposed of in a box in the 1st floor administrative office.
 - d. Restock sonde kits of supplies used in the field.
 - e. Quality control and assurance (QAQC) data following the code in the [github repository](#).

10 Waste Disposal

1. Dispose of discarded pH filling solution and Quick Cal solution (See Section 9.3 and Sonde Calibration SOP).

11 References

Data sonde, main unit

- [Aqua TROLL 600 Multiparameter Sonde](#) [Quick Start Guide](#) [Manual](#)

Data sonde, added on components

- [Aqua TROLL Temperature-Conductivity Sensor](#)
 - [Aqua TROLL RDO Sensor \(Includes RDO X Cap\)](#)
 - [Aqua TROLL Chlorophyll a Sensor](#)
 - [Aqua TROLL pH / ORP Sensor](#)
 - [Aqua TROLL Wiper](#)
 - [Twist-Lock Backshell Hanger](#)
- | |
|--|
| Instruction Sheet
Instruction sheet
Manual
Manual |
|--|

Data sonde accessories

- [Quick Cal solution](#)
 - [Aqua TROLL Replacement Desiccant](#)
 - [pH Filling Solution](#)
- | |
|---|
| Safety Data Sheet
Manual
Manual |
|---|

Spec Sheet for Data Sonde and Sensors

Data Sonde Instruction Sheets: All hard copies of the instruction sheets that came with the data sondes and sensors are kept in a binder in Rm 26 (not all of the sheets are available online).

12 Appendices

- A. Items in the gray floating tray for servicing the sonde:
 - a. Field Tablet (has a yellow float containing VuSitu app).
 - b. Zipties of appropriate size
 - c. Socket wrench
 - d. $\frac{15}{16}$ inch open-end wrench
 - e. Adjustable wrench
 - f. Extra pins and bolts for housing
 - g. Towel / cloth for wiping things down.
 - h. Dish scrubbing pad
 - i. Scrub brush
 - j. Shucking Knife
 - k. 500 ml empty container with sealable top
 - l. wire cutters
 - m. Sonde supply box
 - i. High vacuum grease
 - ii. RDO sensor Calibration sponges
 - iii. Desiccant tube
 - iv. Hex driver
 - v. allen key
 - vi. sonde wipers
 - vii. Alcohol pads
 - viii. D batteries (minimum of 2, Energizer or Duracell only). They should be in a plastic bag to distinguish from old batteries.
 - ix. Paper towels (for collecting discarded pH filling solution).
 - x. Q-tips or cotton buds

- B. Removing fouling organisms from sonde (see Sonde Assembly, Deployment, Storage SOP)
 - a. pH sensor -
 - i. To remove crystalline deposits:
 1. Soak the sensor with warm water and mild soap and rinse with fresh water to clean. Soak the sensor in 5% HCl solution for 10 to 30 minutes. If deposits persist, alternate soaking in 5% HCl and 5% NaOH solutions.
 - ii. To remove oily or greasy residue:
 1. Soak the sensor with warm water and mild soap and rinse with fresh water to clean. Methanol or isopropyl alcohol may be used for short soaking periods, up to 1 hour. ***Do not soak the sensor in strong solvents, such as chlorinated solvents, ethers, or ketones, such as acetone.***

- iii. To remove protein-like material, or slimy film:
 1. Soak the sensor with warm water and mild soap and rinse with fresh water to clean. Soak the sensor in 0.1 M HCl solution for 10 minutes and then rinse with deionized water.
- b. RDO sensor:

If extensive fouling or mineral buildup is present, soak the sensor in vinegar for 15 minutes, then soak in deionized water for 15 minutes. ***Do not use organic solvents—they will damage the sensor cap. Do not remove the sensor cap when rinsing or brushing.***
- c. Temperature/Conductivity sensor:
 - i. To remove crystalline deposits:
 1. Clean the sensor face with warm water and mild soap. Use a soft brush to gently clean the sensor pins and temperature button. Ensure removal of all debris around the base of the pins and button. If crystalline deposits persist, soak in 5% HCl for 10 to 30 minutes followed by warm soapy water and soft brushing. If deposits persist, alternate soaking in 5% HCl and 5% NaOH solutions followed by warm soapy water and soft brushing.
 - ii. To remove oily or greasy residue:
 1. Clean the sensor face with warm water and mild soap. Using a soft brush, gently clean the sensor pins and temperature button. Ensure removal of all residue around the base of the pins and temperature button. Isopropyl alcohol may be used for short soaking periods, up to one hour. ***Do not soak in strong solvents such as chlorinated solvents, ethers or ketones (such as acetone).***
 - iii. To remove protein-like material, or slimy film:
 1. Clean the sensor face with warm water and mild soap. Using a soft brush, gently clean the sensor pins and temperature button. Ensure removal of all material/film around the base of the pins and temperature button. Soak the sensor in 0.10% HCl for 10 minutes and then rinse thoroughly with distilled water.

END OF SOP

Appendix O – Monthly HOBO Maintenance SOP

1 Purpose

The purpose of this SOP is to provide concise guidance and methodology for maintaining the HOBO temperature loggers on a monthly basis to accurately collect year round temperature data.

2 Scope

This SOP is pertaining to the EPA Long Island Sound Study funded Oyster Health project that is incorporating water quality metrics to understand host-pathogen-environment relationships.

3 Definitions/Acronyms

4 Safety Precautions

All survey team members will wear appropriate clothing dependent on weather conditions including but not limited to waders, rubber boots or protective footwear, gloves, hats, sunglasses, long sleeve shirts, and pants. Thick protective gloves (e.g garden gloves) should be worn when handling fouling organisms. Team members will wash hands thoroughly after field trips end. A first aid kit will be present for any injury. Extra water will be provided to avoid dehydration or heat stroke. Team members will take regular breaks when needed.

Exercise weather-appropriate field safety measures by monitoring conditions before and during the trip. Do not perform fieldwork during dangerous conditions (e.g. lightning, extreme winds, extreme floods). Do not visit field sites alone (use buddy system). Inform PIs of dates and times of fieldwork. Confirm safe return to the lab. At intertidal sites, perform procedures during low tide. At subtidal sites, divers are to follow NOAA diving regulations according to the instructions of the lab diving coordinator (Barry Smith, barry.smith@noaa.gov)

5 Reagents/Media

6 Supplies/Equipment

1. Field tablet (containing HOBOconnect app)
2. Robust zip-ties for securing HOBO to sonde or housing(e.g., 6-12 inches and 18-24 inches)
3. Scrub brush
4. Shucking knife
5. First aid kit
6. Wire cutters
7. Hand sanitizer
8. Spare HOBO battery (Murata brand CR2477 premium lithium 3V coin cell batteries).
9. Spare O-rings for HOBO data logger. ([MX2203 Replacement O-ring](#)).

7 Quality Control

All team members will be trained to complete all field tasks, including training on data entry requirements for each specific task. Environmental monitoring data will be regularly checked for drift.

8 Preparation (At the lab)

1. Ensure that all SOPs and field notebooks are available offline on the field tablet.
 - a. Monthly Sonde Maintenance
 - b. Monthly HOBO Maintenance
 - c. Water Sampling and Chemistry
 - d. Sonde Sensor Calibration
 - e. Sonde Field Notebook
 - f. Site specific field notebook
2. Ensure that the field tablet is fully charged.

9 Procedures

1. Setup and deployment:
 - a. *Note: The following steps occur when the HOBO is out of the water.*
 - b. Log into the Field tablet
 - c. Enable bluetooth in the tablet settings.
 - d. Open the HOBOconnect app.
 - e. To activate the temperature logger, firmly press the magnetic button (top circular part of the protective boot with "HOBO" embossed on it), holding it for 1 second against the logger (Figure 1). The app will detect the instrument on the tablet given the device is within range.

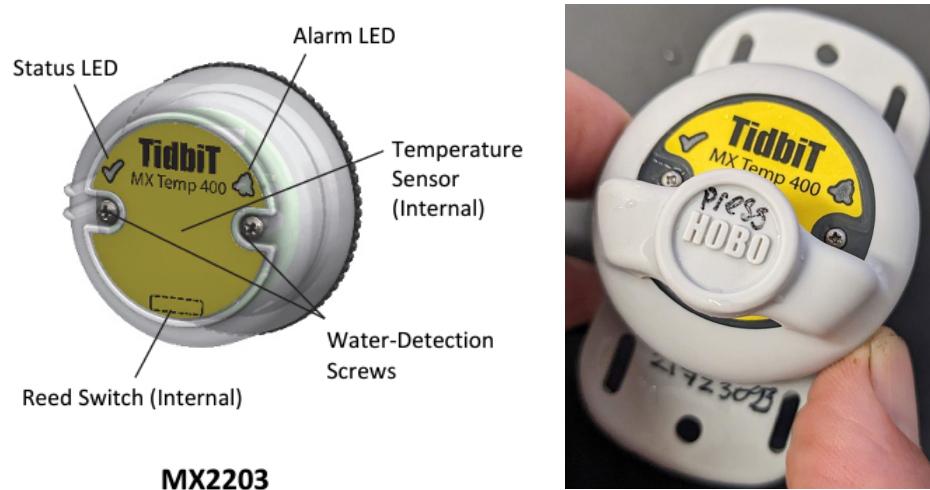


Figure 1: TidBiT MX Temp 400 HOBO. Left: Instrument parts. Right: HOBO mounted in its protective boot. ("Press" indicates area to press when waking up the HOBO).

- f. An icon for the specific HOBO device will appear in the app. Click on the icon to connect it to the tablet.

- g. To create a new log session, click the “Start Logging” icon and follow the prompts to set up the session. Settings should be as follows:
 - i. Logging Interval = 10 min;
 - ii. Start Logging = manual (starts on save) or set a date and time, if appropriate;
 - iii. Stop Logging = Never Stop (overwrites old data);
 - iv. Logging Mode = Fixed Logging Mode;
 - v. Power Saving Mode = Bluetooth Always Off;
 - vi. Show LED = On (swipe right in the app);
 - vii. Alarms = Not Configured.
- h. Write down the settings in the field notebook.
- i. Some notes (from the manual) about the Status and Alarm LEDs:
 - i. The Status LED blinks green every 4 seconds when the logger is logging (unless Show LED is disabled when configuring the Logger).
 - ii. If the logger is waiting to start logging because it was configured to start “On Button Push” or with a delayed start, it blinks green every 8 seconds.
 - iii. Both the Status LED and the Alarm LED blink once when you press the button to wake up the logger, or blink four times when you press the button to start or stop logging.
 - iv. If you click the LED icon in the app, both LEDs are illuminated for 5 seconds.
- j. Initial Deployment:
 - i. Intertidal sites:
 1. During field season (March–November), HOBOs are attached to rebar anchoring spat collector bags to the seabed. Spat collector bags are located within the oyster bed at the lowest zone or fringe. Attach the HOBOs using small zip-ties threaded through the top and bottom rubber lip of the HOBO (Figure 2).
 2. During off season (December–February), all HOBO loggers are attached to sonde housings. Attach HOBOs using large zip ties threaded through the tip and bottom of the rubber lip of the HOBO and secure the device below mean low water around the sonde housing.



Figure 2: Depiction of where to thread the zip-ties

- ii. Subtidal sites (using NOAA Divers):
 - 1. At Gold Star Beach (from Cornell Extension FLUPSY):
 - a. Use the long brush to clean the inside and outside of the PVC housing, making sure the vent holes are clear to allow water flow.
 - b. Place the sonde plus attached HOBO (zip-tied at top of sonde) inside the PVC housing with the probes facing downwards.
 - c. Clip the tether from the anchor onto the top of the data sonde.
 - d. Pass a zip-tie through one of the PVC vent holes and out another (about half way up the sonde) and use it to secure the sonde tight against the inside of the PVC housing.
 - e. Lower the mushroom anchor to the seabed and fasten the line on a cleat.
 - 2. Retrieving deployed HOBO:
 - a. Intertidal sites:
 - i. Wade to the location of the HOBO (See 9.1.j) and remove the HOBO using a wire cutter. Be sure that you are cutting the zip-ties and not the HOBO itself.
 - b. Subtidal sites:
 - i. For subtidal sites, HOBO loggers are always attached to sonde housings/ mushroom anchors. See Monthly Sonde Maintenance SOP for more details on retrieving sonde housing at subtidal sites.
 - ii. Laurel Hollow:
 - 1. A single HOBO is zip-tied to the top of a data sonde, which is deployed on a mushroom anchor marked by a NOAA buoy. SCUBA divers will need to remove the data sonde and bring it to a shore or boat support party, as follows:
 - a. Unclip the parachute cord that loops from the top of the data sonde around the shank of the anchor.
 - b. Use wire cutters to cut a large zip-tie that secures the data sonde to the PVC housing (the one that passes through the vent holes of the PVC housing, not the zip-ties holding the PVC housing to the anchor itself).
 - c. Remove the data sonde from the PVC housing (it also has a HOBO data logger attached near its top end). Return the sonde + HOBO to the support boat or shore party.
 - iii. Gold Star Beach: Access Cornell Extension's FLUPSY . Pull up the 25 pound mushroom anchor from the seabed and place it on the dock. The HOBO is zip-tied to a data sonde attached to the anchor.
 - 3. Downloading data
 - a. Data can be downloaded once the HOBO logger is out of the water.
 - b. Log into the Samsung tablet . Enable Bluetooth in the tablet Settings.
 - c. Open the HOBOconnect app.
 - d. Wake up the temperature logger by firmly pressing the magnetic button (top circular part of the protective boot with "HOBO" embossed on it), holding it for 1

- second against the logger (Figure 1). The app should detect the instrument on the tablet.
- e. On the tablet, connect to the HOBO by clicking its icon.
 - f. Click the Download Data icon.
 - g. When “Download Complete” is displayed, click Export & Share.
 - h. When “Export successful” is displayed, click Share.
 - i. Save the file to the site specific folder on the shared drive (shortcut to *Data_Sondes* folder; full path: *Project Planning / Data Management / Field Data / Data_Sondes/HOBO data*)
 - j. Once saved, the app will return to the home screen (it should indicate “Logging” is still in progress near the top).
 - k. The remaining battery level should display in the app home screen. If $\leq 50\%$, stop the logging session (icon on app home screen) and change the battery (see 9.4—this should be a rare event). If $> 50\%$ disconnect the app (arrow at the top left of the screen) and redeploy the HOBO (see below).
 - l. On return to the lab, sync the downloaded data (see Section 10).
- 4. Changing the battery**
- a. Battery changes must occur when the HOBO has been retrieved, surfaced, and brought to shore. The HOBO must be out of the water.
 - b. Pop the data logger out of its protective boot.
 - c. Clean, rinse and thoroughly dry the data logger.
 - d. Undo the back of the logger by pushing down on the back and rotating the cover counterclockwise (align the unlock icon with the double-ridge on the side of the logger). (Figure 3)

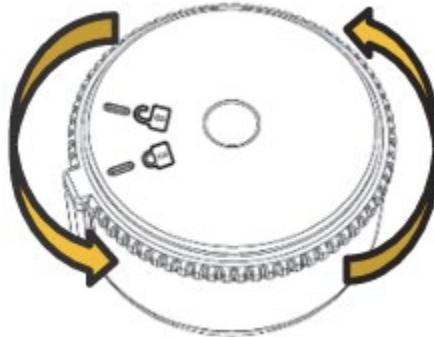


Figure 3: Counterclockwise rotation to remove back of HOBO logger.

- e. Lift the cover off the logger and remove the battery.
- f. Insert a new battery (CR2477 lithium 3V) with the positive side facing up.
- g. Make sure the O-ring is clean, seated properly and free of cracks. If damaged, replace the O-ring as follows:
 - i. Spread a small dot of silicone-based vacuum grease on the new O-ring, making sure the entire O-ring surface is completely covered in grease.
 - ii. Place the O-ring on the cover and clean off any debris. Make sure the O-ring is fully seated and level in the groove and not pinched or twisted. This is necessary to maintain a waterproof seal.
- h. Place the cover back on the logger, lining up the unlock icon with the double-ridge on the side of the logger case. Make sure the cover is level as it is placed on the logger case to ensure the battery terminal maintains its proper position.

- i. While pushing down on the cover, rotate it clockwise until the tab is aligned with the double-ridge in the logger case (Figure 4). Rotate the locked icon so that the icon moves from the unlocked to locked position (Figure 4). When the cover is properly positioned, the tab and the locked icon will be aligned with the double-ridge in the logger as shown.

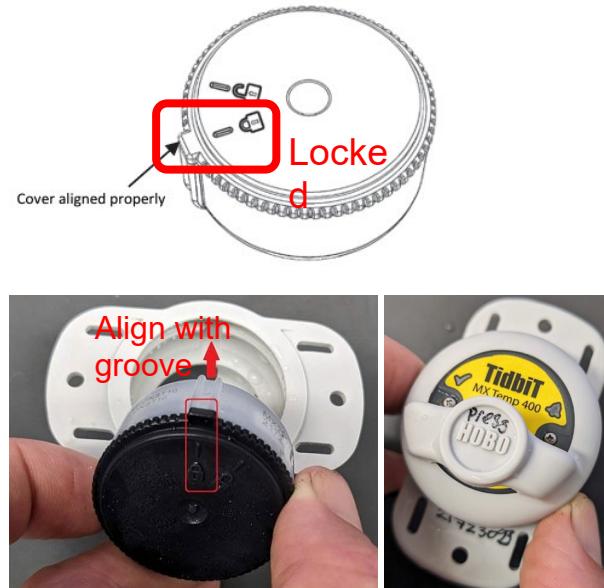


Figure 4: Aligning the cover and logger case to close the battery compartment.

- j. Place the logger back in the protective boot, making sure the double-ridge on the logger case slides into the groove inside of the boot. Viewed from the front, the writing on the logger should be visible and horizontally aligned inside the boot.
 - k. Use zip-ties to attach the logger + protective boot onto its deployment structure (rebar, around sonde housing, etc). Ensure the back of the protective boot is against something or covered to prevent the logger popping out.
- 5. Checking the accuracy of temperature sensor (Ice bath method), See Appendix A.**
This only needs to be performed in the event that the recorded temperatures seem to be drifting or otherwise inaccurate.

10 Lab return

1. Rinse all tools (wrenches, pliers, wire cutters) with fresh water, dry them with a towel, and spray with WD40 (kept on field gear shelves).
2. Take the Samsung tablet to the library and connect it to the WiFi in order to sync the shared Google drive (contains the downloaded data files from the sonde and HOBO).
3. Quality control and assurance (QAQC) data following the [github repository](#).

11 References

[HOBO manual](#)[HOBO TidbiT MX Temperature 400' Bluetooth enabled data logger](#)[HOBO TidbiT MX Temp 400 \(MX2203\) and Temp 5000 \(MX2204\) Logger Manual.](#)[HOBO's ice bath test protocol](#)

12 Appendices

A. Checking the accuracy of temperature sensor (Ice bath method)

- a. Note: HOBO temperature sensors are factory-calibrated. There is no option for a user to calibrate the sensor, but its accuracy can be tested using an ice bath test, as recommended by the HOBO manufacturer.
- b. Fill a 5-gal bucket with ice (the ice machine in the basement of Milford Lab, Building 1).
- c. Pour all the ice into a cooler (e.g., the oyster project's 38 quart cooler). It should fill the cooler at least half way. If not, add some more ice.
- d. Pour tap water into the cooler, adding just enough to make a thick slurry of icy water.
- e. Set the HOBO to record every 1 minute (see above), then place it in the ice (hover it mid-depth within the watery ice slurry).
- f. Close the cooler lid and leave the HOBO to record for at least 90 minutes (it takes a while for the HOBO to cool down, and you want a good period of stabilized low temperature readings to check consistency).
- g. Retrieve the HOBO and download the data (see section 3 above).
- h. Examine the data in Excel. The temperature should have declined steadily, then stabilized very near 0°C. Select 30 readings (a 30 minute period) from the stabilized records (ignore the last few records in the log, which may have recordings after the HOBO was removed from the ice). From the 30 readings, calculate the mean, minimum, and maximum. The mean should be within 0.25°C of 0°C (the manual states an accuracy of plus or minus 0.25°C at 0°C). Check the minimum and maximum values for out-of range fluctuations. Note that minor changes in the true temperature (assumed to be 0°C) may be caused by dissolved salts in the ice and water (both tap water), and by changes in atmospheric pressure. These effects are considered negligible.
- i. Save the Excel file on the project shared Google drive under the folder Fieldwork/Data sondes and HOBOs/Ice Bath Tests

END OF SOP

Appendix P – Sonde Sensor Calibration SOP

1 Purpose

The purpose of this SOP is to provide concise guidance and methodology for calibrating the Aqua Troll 600 sonde to accurately collect temperature, salinity, pH, RDO, and chlorophyll-a data of quality.

2 Scope

This SOP is pertaining to the EPA Long Island Sound Study funded Oyster Health project that is incorporating water quality metrics to understand host-pathogen-environment relationships.

3 Definitions/Acronyms

4 Safety Precautions

All team members will wear appropriate clothing dependent on weather conditions including but not limited to waders, rubber boots or protective footwear, gloves, hats, sunglasses, long sleeve shirts and pants. Thick protective gloves (e.g garden gloves) should be worn when handling fouling organisms. Team members will wash hands thoroughly after field trips end. A first aid kit will be present for any injury. Extra water will be provided to avoid dehydration or heat stroke. Team members will take regular breaks when needed.

Exercise weather-appropriate field safety measures by monitoring conditions before and during the trip. Do not perform fieldwork during dangerous conditions (e.g. lightning, extreme winds, extreme floods). Do not visit field sites alone (use buddy system). Inform PIs of dates and times of fieldwork. Confirm safe return to the lab. At intertidal sites, perform procedures during low tide. At subtidal sites, divers are to follow NOAA diving regulations according to the instructions of the lab diving coordinator (Barry Smith).

5 Reagents/Media

1. Bottle of distilled water (1 L) for rinsing sonde and probes.
2. In-Situ [pH filling solution](#) for replacing fluid inside pH sensor (SKU 0056900). In Garage.
3. In-Situ [Quick Cal solution](#) for calibrating pH sensor (SKU 0033250). Fridge, Rm 29.

Refer to note under Section 8 (Quality Control).

6 Supplies/Equipment

Field Tablet (containing VuSitu app).

Robust zip-ties (e.g., 24 inch) for securing the sonde to the PVC housing at subtidal sites.

1516 inch socket wrench

1516 inch open-end wrench

Adjustable wrench

Sonde supply box

High vacuum grease

RDO sensor Calibration sponges

Extra pins and bolts for housing

Desiccant tube

Screw drivers
 Hex driver
 Allen key
 Sonde wipers
 Alcohol pads
 D batteries (minimum of 2, Energizer or Duracell only).
 Paper towels (for collecting discarded pH filling solution).
 Towel / cloth for wiping things down.
 Dish scrubbing pad
 Scrub brush
 Shucking knife
 Q-tips or cotton buds
 500 ml empty container with sealable top
 First aid kit
 Hand sanitizer
 Wire cutters

7 Equipment

8 Quality Control

1. All team members will be trained to complete all field tasks, including training on data entry requirements for each specific task. Environmental monitoring data will be regularly checked for drift and sensors will be calibrated when necessary.
2. Reagent care:
 - a. Quick Cal solution: Use a new (unopened) bottle of Quick Cal solution each field trip for calibrating the pH probe. Keep in a dark bottle (it is light sensitive). Quick Cal can be kept unopened in the fridge for ~6 months (check expiration date on bottle). Use a fresh, unopened bottle for each calibration (once opened it lasts ≤ 7 days in the fridge). Before opening and using the solution (e.g., while driving to the field site), let it come to ambient temperature. This helps the probe stabilize during the calibration process.
 - b. pH filling solution: Store at room temperature (or fridge, but not frozen or at high temperatures; In-Situ tech support, 7/7/23). It lasts for ~2 years (check expiration date on bottle).

9 pH Sensor

1. *The pH sensor is recommended to be calibrated every 10-12 weeks. The reference filling solution inside the pH sensor should be replaced between deployments or when measurements begin to drift.* This may be a frequency of every 5-6 months or when the sensor fails to calibrate within the acceptable slope and offset; sensor readings vary; readings during calibration at pH 7 are greater than +30 mV or less than -30 mV; or the sensor is slow to respond.
2. Unscrew the copper guard from the sonde, as well as the black cap that is screwed into the top end of the copper guard.
3. Rinse the probes, copper guard, and black end cap with distilled (or tap) water. Dry all pieces using a towel or cloth. *Don't wipe the sensor surfaces.* If necessary, debris can be gently removed following the 'Monthly Sonde Maintenance' SOP.
4. Calibrate the pH sensor

- a. Rinse the probes with Quick Cal solution (Figure 1):
 - i. Screw the copper guard onto the sonde in the calibration/storage position.
 - ii. Connect the data sonde with the VuSitu app on the Samsung tablet (see 'Monthly Sonde Maintenance' SOP for instructions), then select Calibration / Quick Cal Calibration. The calibration steps will require the probes to be rinsed with Quick Cal (rinse it twice) before refilling with Quick Cal for the actual calibration.
 - iii. To rinse sensors with calibration solution:
 - Hold the sonde with the probes facing upwards.
 - Unscrew the black end cap from the top of the copper guard
 - Pour enough Quick Cal solution to fill the probe well (created by copper guard) about 2/3 of the way up.
 - Lightly screw on the black end cap of the copper guard and rinse the sensors and well by swirling the Quick Cal solution.
 - Remove the black end cap and pour the Quick Cal solution into a discard container.
 - Repeat the previous rinsing step, giving the probes a second rinse of Quick Cal solution (keeping enough Quick Cal for the calibration step).

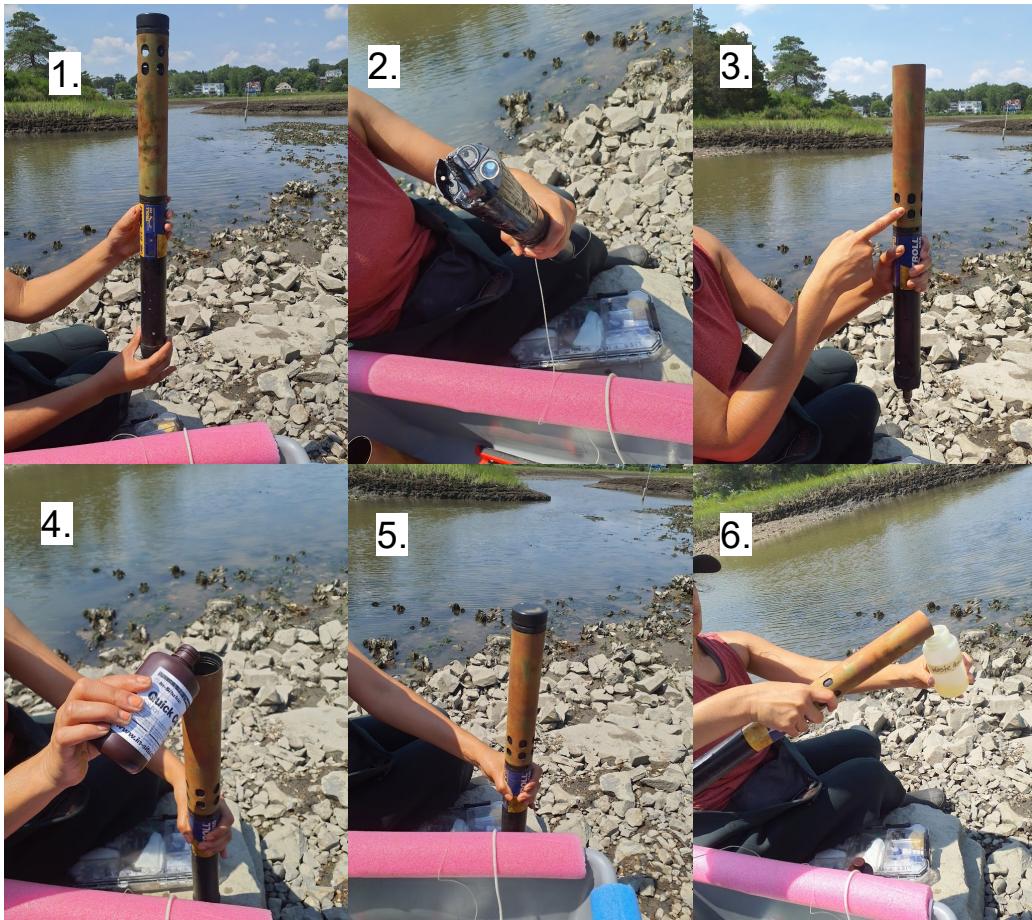


Figure 1. Rinsing the sensors with Quick Cal solution. 1. Holding sonde with probes facing upward to connect to bluetooth. 2. removing copper guard to expose sensors. 3. Placing copper guard in storage position. 4. Filling with Quick Cal solution. 5. Capping copper guard and swirling solution. 6. Discarding Quick Cal solution into a waste container.

- b. After rinsing the probes, refill the probe well with fresh Quick Cal solution and calibrate the pH sensor by following the steps in the app (Figure 2):

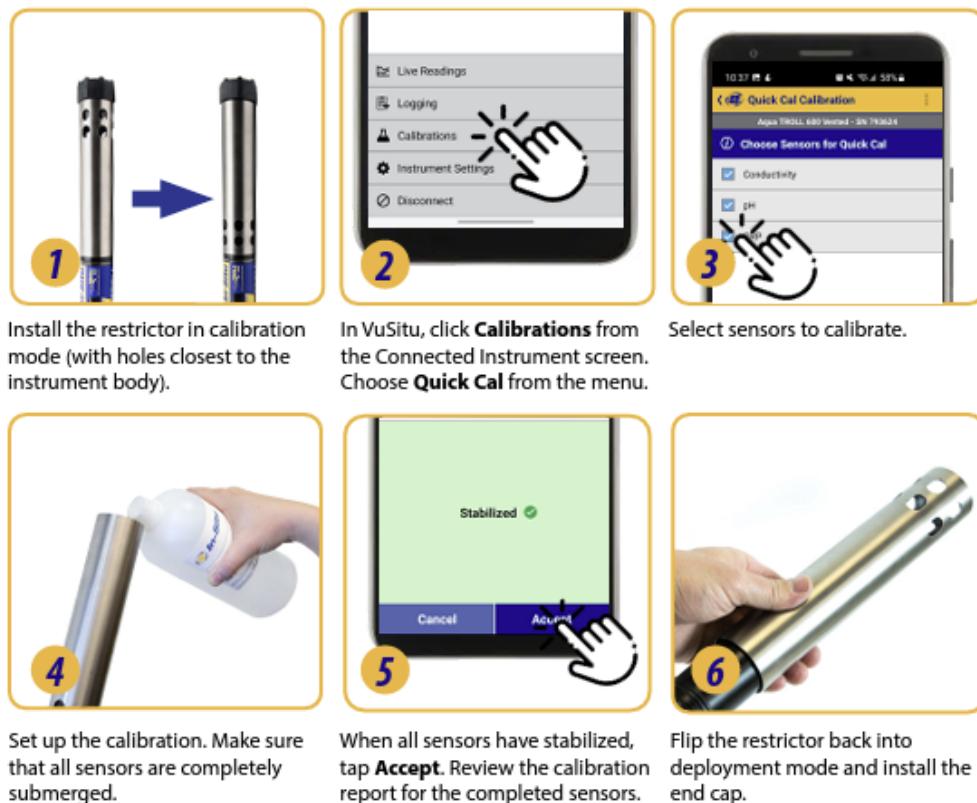


Figure 2. Steps for calibration of the pH sensor in the VuSitu app.

5. Reference filling solution:

- The sensor fill solution has a shelf life of 2 years. Replace the fill solution every 5 to 6 months or when:
 - The sensor fails to calibrate within the acceptable slope and offset range.
 - Sensor readings vary.
 - Readings during calibration at pH 7 are greater than +30 mV or less than -30 mV
 - Sensor is slow to respond.
- Remove the pH sensor from the sonde:
 - Rotate the wiper arm (the little brush that cleans the sensors) into a position so that it does not obstruct the top of the pH probe. (Note: each type of probe is labeled on its side with raised lettering).

- ii. Use the longest of the three types of sonde screwdrivers (hex driver) to undo the screw that secures the pH probe to the sonde. The screw will unscrew from the sonde housing, but it will stay attached to the probe itself (i.e., it will not fall out and get lost). (Figure 3)



Location of screw that attaches the pH probe to the sonde (yellow arrow), and the small indent that can be used for gently prying the probe from the sonde (blue arrow).

Figure 3: Location of the screw to remove the pH sensor from the sonde and the appropriate screwdriver needed to perform removal.

- c. Once the screw is undone, insert the same screwdriver into a small hole at the base of the probe (at the junction between the base of the probe and the sonde; not at the sensor end). Gently push upwards until the pH probe releases from the sonde. Then lift the pH probe from the sonde, making sure that the electronic interfaces remain dry.
- d. Replace the pH filling solution in the probe using the instructions below (Figure 4).

Note 1: Be careful not to lose the reference junction (little screw top on the probe's filling solution reservoir) - unscrew it over the gray tray. Keep it moist and clean.

Note 2: Before putting the reference junction back on, make sure the O-ring is cleaned and vacuum greased. Tap the probe lightly before resealing the filling solution reservoir to release any trapped air bubbles.



Figure 4: Steps to replace the reference filling fluid

e. Replacing the reference junction:

- i. Replace the junction when the sensor fails to calibrate with a reasonable slope and offset, even after you have replaced the filling solution.
 1. Unscrew the reference junction and discard.
 2. Replace the filling solution and screw in a new reference junction.
 3. Soak for 15 minutes, then calibrate the sensor.

f. Reattach the pH probe onto the sonde:

- i. Make sure the pH probe's O-ring is clean and dry.
- ii. Apply a little vacuum grease (tubes in the red box) to the O-ring.
- iii. Gently push the probe into its original sonde slot, making sure everything is aligned properly (check alignment at the sensor end too).
- iv. Gently tighten the screw that secures the probe to the sonde using the longest of the three types of sonde screwdrivers (red box). It does not need to be especially tight (beware of cracking the plastic housing).

10 Conductivity sensor

1. *The conductivity sensor should be calibrated every 12 months.*
2. If there is reason to believe that the sensor needs calibrating, use the Quick Cal solution and follow the instructions for Quick Cal calibration of the pH sensor (Section 9.4).
 - a. *In the VuSitu Quick Cal calibration steps, make sure that the conductivity sensor is selected for calibration.*

11 Temperature sensor

1. The sonde manual (p. 45) recommends that the conductivity sensor should be calibrated “only when required by user protocol”.
2. There is no user-calibration option for the temperature sensor. It is possible, however, to check the accuracy of the temperature sensor using the [ice bath method](#) also used for recommended by HOBO temperature loggers (In-Situ tech support, May 2023).

3. It is also possible to check the accuracy of the temperature sensor by taking side-by-side temperature readings to compare sonde data against another instrument (e.g., HOBO temperature logger) that was recently ice bath tested.

12 RDO Sensor

1. *The RDO sensor should be calibrated every 12 months depending on site and storage conditions.*
2. To calibrate the RDO sensor, use the ‘RDO 100% saturation with water saturated air method’. (Figure 5)
 - a. Round sponges are in the sonde maintenance kit box which can be found in the LISS field gear station in the garage of the NEFSC.
 - b. Remove the cap from the copper guard. Place the copper guard (aka restrictor) in the calibration/storage position (holes facing screen and centered on the instrument)
 - c. Lightly dry the RDO sensor before calibration using a cotton swab.
 - d. Saturate the small round sponge with water. Seawater or freshwater okay. The water only serves to saturate the air in the copper guard.
 - e. Place the sponge on the guard cap and *loosely install the cap allowing for airflow. If the cap is too tight it will disrupt the calibration.* Be sure to keep the face of the sensor dry.
 - f. Leave the sponge in the copper guard for 5 minutes.*Do not wait beyond 5 minutes before performing the calibration. Condensation may start to form on the RDO sensor, which interferes with calibration.*
 - g. Open the VuSitu app, click calibration, RDO sensor, and follow the instructions to complete the calibration.



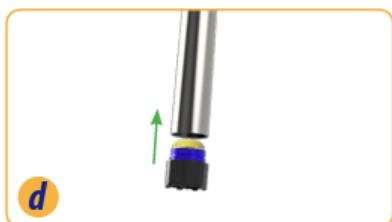
Place the restrictor in calibration mode (holes near center of instrument).



Saturate a small sponge with water.



Place the sponge on the restrictor cap.



Loosely install the end cap, keeping the sensor face dry and allowing for air flow.



Leave sponge in restrictor for five minutes.



Follow the instructions in VuSitu to finish calibration.

Figure 5 : Steps to calibrate the RDO sensor.

13 Chlorophyll-a sensor

1. *The chlorophyll-a sensor should be calibrated every 12 months.*
2. If the sensor needs calibrating, reset the zero point by performing a calibration with deionized water:
 - a. Remove the copper guard of the sonde.
 - b. Rinse the guard and sensors with distilled water.
 - c. Screw the copper guard back on in calibration mode (screw it on by the threads at the vent hole end of the guard) and invert the sonde (sensors facing up).
 - d. Fill the sensor chamber with distilled water to the bottom of the cap end threads.
 - e. Screw the end cap onto the copper guard. *It is important that no light is able to enter the sensor chamber.*
 - f. Connect the sonde with the VuSitu app on the Samsung tablet.
 - g. Select Calibrations from the menu.
 - h. Choose the Chlorophyll option and follow the instructions.

14 Waste Disposal

1. Discarded pH filling solution should be poured onto a paper towel while in the field and disposed of in the trash on return to the lab (as per In-Situ instruction sheet).
2. In the field, discarded Quick Cal solution should be poured into a waste container and sealed. On return to the lab, it should be poured down the sink with running tap water (as per instructions from In-Situ tech support, 7/7/23).

15 References

Data sonde, main unit

- [Aqua TROLL 600 Multiparameter Sonde](#) [Quick Start Guide](#) [Manual](#)

Data sonde, added on components

- | | |
|--|-----------------------------------|
| • Aqua TROLL Temperature-Conductivity Sensor | Instruction Sheet |
| • Aqua TROLL RDO Sensor (Includes RDO X Cap) | Instruction sheet |
| • Aqua TROLL Chlorophyll a Sensor | Manual |
| • Aqua TROLL pH / ORP Sensor | Manual |
| • Aqua TROLL Wiper | |
| • Twist-Lock Backshell Hanger | |

Data sonde accessories

- | | |
|--|-----------------------------------|
| • Quick Cal solution | Safety Data Sheet |
| • Aqua TROLL Replacement Desiccant | Manual |
| • pH Filling Solution | Manual |

Spec Sheet for Data Sonde and Sensors

16 Appendices

- A. Shelf life of QuickCal Solution
 - a. Per In-Situ - Shelf life of unopened bottles, in cold dark places is only 4 months and after opening 7-21 days.

END OF SOP

Appendix Q – Dissolved Inorganic Carbon Measurements (Water Chemistry)

By: Shannon Meseck and Genevieve Bernatchez

1 Purpose

The purpose of this SOP is to provide concise guidance and methodology for measurement of dissolved inorganic carbon from discrete water samples.

2 Scope

This SOP is pertaining to the EPA Long Island Sound Study funded Oyster Health project that is incorporating water quality metrics to understand host-pathogen-environment relationships.

3 Definitions/Acronyms

Dissolved Inorganic Carbon (DIC)

Certified Reference Materials (CRM)

4 Safety Precautions

PPE: Lab coats, nitrile gloves, safety goggles

Gloves should be worn when working mercuric chloride ($HgCl_2$). No hazardous waste may be washed down the drain. Please place hazardous waste in the appropriately marked containers in the satellite accumulation area

5 Reagents/Media

Certified Reference Materials (Dickson Lab CO_2 CRM Batch 191 and 157)

mili-Q water

6 Equipment

Apollo SciTech DIC analyzer Model AS-C5

7 Procedures

- Turn the DIC for about 30 minutes before using it
- Turn the main power on
- Turn the black on/off button
- Turn the nitrogen on (flow must be low), do not open the valve all the way.
- Computer is usually on standby, just press the power button to wake up the computer
- Click on the new icon for DIC analysis.
- Facing the machine, the left line is for the reagent (8.5% Phosphoric acid solution). O-phosphoric acid is 85% concentrated. The bottle holds 500 mL of solution. To make it

you need to dilute the original by 10X. To 450 mL of DI or Milli-Q water add 50 mL of O-phosphoric acid. Mix and put in an amber bottle.

- The right line is for the DI water or samples
- On the computer, highlight control, click on connect/disconnect to connect to the machine
- Highlight Batch process
- Under sample name enter the name of the samples, enter sample volume
- Calibration curve (done every day).
 - Dry sodium carbonate to constant weight in an oven at 280C for more than 2 hours and store in a desiccator. Make sure you use 99.95% pure sodium carbonate
 - Boil Milli-Q water to remove carbon dioxide for 30 minutes, Cool water in fume hood by bubbling with nitrogen. Make sure the solution is cooled to room temperature 20C
 - Weigh the flask (wear gloves otherwise fingerprints will be on weight)
 - Weigh the appropriate amount in the flask approximately 25 mg of sodium carbonate. You want a final concentration at around 2500 umol/kg. Always write down what you weigh. Weigh flask again and tare the scale
 - Transfer cooled nitrogen bubbled mili-Q water to the flask
 - Shake for about 30 seconds, added more mili-Q water to the meniscus
 - Weigh the flask to get the total amount of mili-Q water, record the weight
 - Put the stopper on the flask and invert the flask 30 times
 - Nitrogen purge flask and keep at 20C until you run the standard
 - You need to make one standard everyday
- Before running the sample, move waste tubing into the waste container.
- First sample run is DI water.
- Then run the calibration curve.
- To make the calibration curve stick the standard on the instrument line. Vary the amount inject from as follows

Sample name	sample number	Volume
Standard1	STD 1	1.0
Standard1	STD 2	0.8
Standard1	STD 3	0.6
Standard1	STD 4	0.4
Standard1	STD 5	0.2

When the standards are used, they must be immediately filled with nitrogen. Remake standard daily.

- The CRM bottle, if you need to open a bottle, use the wrench, slowly grip the top on both sides where you have ridges, and slowly try to loosen up the cap. The CRM does have 1% mercury chloride in it
- Always put the waste in the brown bottle marked for CRM waste

- Run a minimum of 2 CRM during the day
- For the CRM, wrap the sample tube with a kimwipes to avoid the grease touching the tube and use the funnel
- To start a sample, highlight the line and click on start measurement
- When analyzing samples due a volume of 1.0 ml, including the 2 CRMS
- To avoid mixing samples, press on start measurement before entering the tube in the sample, it will suck a little air in between samples. Also make sure to use kimwipes on tubing before transferring
- Run the CRM protocol at the end and the beginning of running all samples
- After all samples are done, but both lines in DI water and run it as a sample
 - If not running instrument long term (>1 week) after run instrument with water take both tubing out into air and run the cycle through
- On the computer, highlight control, click on connect/disconnect to connect to the machine
- Answer yes to disconnect. Let it run through its cycle
- After complete turn off instrument and nitrogen gas
- Enter the data in the excel spreadsheet for calculations

Appendices

If issues while running, immediately contact Shannon Meseck (203)882-6531 or cell (203) 892-8877 or George Sennefelder (203)882-6548 or cell (203) 270- 1318 or 203-788- 1071

Tubing information

Cole-Parmer PTFE Tubing, 1/16"ID x 1/8"OD, item # 06605-27 sample tube
Cole-Prmer PTFE, 1/32" ID x 1/16 " OD; 25 ft 06407-41 acid tube

END OF SOP

Appendix R – Alkalinity Measurements (Water Chemistry)

By: Shannon Meseck and Genevieve Bernatchez

1 Purpose

The purpose of this SOP is to provide concise guidance and methodology for measurement of total alkalinity from discrete water samples.

2 Scope

This SOP is pertaining to the EPA Long Island Sound Study funded Oyster Health project that is incorporating water quality metrics to understand host-pathogen-environment relationships.

3 Definitions/Acronyms

Certified Reference Materials (CRM)

4 Safety Precautions

PPE: Lab coats, nitrile gloves, safety goggles

No hazardous waste may be washed down the drain. Please place hazardous waste in the appropriately marked containers in the satellite accumulation area

5 Reagents/Media

- Solutions
 - 0.7M Sodium chloride (NaCl*) 40.95 gr/L
 - Bromophenol Blue 500mg/L (50mg/100ml)
 - 0.1M Formic Acid 106µl/25ml
- Mixed Reagents (in 250mL flask)
 - 25ml of formic acid
 - 25ml of bromophenol blue
 - Adjust to 250ml with the sodium chloride solution
- Standards-Same as DIC protocol
- You also need to run Certified Reference Materials (Dickson Lab CO₂ CRM Batch 191 and 157). for accuracy please set up the following.

100%	1.0ml of CRM	0ml NaCl sol*
75%	0.75ml of CRM	0.25ml NaCl sol*
50%	0.5ml of CRM	0.5ml NaCl sol*
25%	0.25ml of CRM	0.75ml NaCl sol*
0%	0ml of CRM	1.0ml NaCl sol* Make 4 0% STD

§ CRM (bottles use for the DIC instrument)

6 Equipment

Metrohm alkalinity titrator (Mettler Toledo T5)

7 Procedures

- To prepare the standards for the spec, add 1ml of mixed reagents and 1ml of standards in falcon polypropylene tubes. Transfer in the small cuvettes
- To prepare the samples for the spec, add 1ml of mixed reagents and 1 ml of samples (dilution needed). Transfer in small cuvettes
- Dilution of the samples (4 fold to start and maybe 5 fold). You can prep the polypropylene tubes prior. For the 4 fold dilution add 0.75ml of NaCL sol*, and 0.25ml of samples. If necessary, for the 5 fold dilution add 0.8ml of NaCL sol*, and 0.20ml of samples. For the 3 fold dilution add 0.67ml NaCl sol* and 0.33ml of samples.
- Read on the spec within 2 hours, use the alkalinity method (590nm)
- Make sure the single cell holders are in the spec, if not unscrewed and put in place. There is one screw in each of the holder cell
- Turn the spec on
- Log in the computer with Shannon's password
- On the computer there is an Advanced Reads icon for the spec, click on it (the icon if not on the desktop can be found in shared documents in Shannon's documents)
- File → open method → use alkalinity method (590nm)

END OF SOP

Appendix S – pH Measurements (Water Chemistry)

By: Shannon Meseck and Genevieve Bernatchez

1 Purpose

The purpose of this SOP is to provide concise guidance and methodology for measurement of total alkalinity from discrete water samples.

2 Scope

This SOP is pertaining to the EPA Long Island Sound Study funded Oyster Health project that is incorporating water quality metrics to understand host-pathogen-environment relationships.

3 Definitions/Acronyms

4 Safety Precautions

PPE: Lab coats, nitrile gloves, safety goggles

No hazardous waste may be washed down the drain. Please place hazardous waste in the appropriately marked containers in the satellite accumulation area

5 Reagents/Media

M-cresol (dye)

6 Equipment

The UV-VIS spectrophotometer

7 Procedures

- Turn the water heater on, by holding down the middle gray button until it turns on (it should be at 20C)
- Make sure the water bath holder cells are in the spec, if not unscrewed and put in place. There is one screw in each of the holder cell. You are using the 10 cm cell holder not the 1 cm cell holder.
- Turn the spec on
- Log in the computer with just username. There is no password. So it is smeseck

- On the computer there is an Advanced Reads icon for the spec, click on it (the icon if not on the desktop can be found in shared documents in Shannon's documents, in pH folder)
- File à open method à use pH method cresol (can be found in share documents in pH folder)
- Each sample will be analyzed 4 times (4 replicates), ALSO each sample goes through 2 rounds one with and one without the cresol. So sample 1 will also be sample 2
- Use the 10 cm glass cell, fill the cell, make it overflow, try to bring the air bubbles near both white caps, to leave a little space for the dye that will be added later. Always clean the cells using kimwipes, especially at both ends where the light beam goes through. NEVER WIPES THE CELL BETWEEN THE TWO READINGS.
- The first cell will be the reference (use H₂O) from the experiment. This sample goes in the back of the spectrometer cell holder.
- Insert the cells into the spec, push them towards the left, and put the reference cell in the back,
- Close the blue lid
- Zero the instrument
- Go to setup on top entire the number of samples in the tab marked samples Hit okay. Remember if you have 5 samples you will have 10 readings. So make sure the double the number of samples for the readings.
- Hit the start button, it will show you the number of samples hit okay.
- Write a file name I usually create a folder with the experiment name then use the date for every time I read
- Load the sample. Make sure the 10 cm cells are dried and the side windows are clean. Only ever use kimwipes on these.
- Press start
- After the reading, remove the sample cells, add 50ul of the m-cresol dye and mix really well, put back in the same orientation in spec and let sit for 4 minutes before reading again. REMEMBER DUE NOT TOUCH THE SIDES OF THE CELL BECAUSE YOU CANNOT CLEAN THEM AGAIN. IF YOU DO YOU NEED TO START WITH A CLEAN NEW SAMPLE WITHOUT THE DYE.

- Use spreadsheet name pH calc spec method to enter the data and get the pH values, the data sheet is in the share documents folder

END OF SOP

END OF QAPP DOCUMENT