1.0 TITLE: Restoration Planning for Hard Clams (*Mercenaria mercenaria*) in Barnegat Bay: Identifying Population Sources and Sinks

QUALITY ASSURANCE PROJECT PLAN

Effective Date: 5/1/2016

Project Duration: March 1, 2016-August 31, 2017

Approved by: _____ 6/21/16
Kathryn Drisco Quality Assurance Officer
U.S. EPA Region 2 — date

Approved by: _____ 6/23/17
Barbara Spinweber, Project Officer
U.S. EPA Region 2 — date

Approved by: _____
Marc Ferko, Quality Assurance Officer
NJDEP Office of Quality Assurance — date

Approved by: _____
Dorina Frizzera, Project Officer
NJDEP Division of Science, Research, and Environmental Health — date

Approved by: _____ 10/25/16
Dr. L. Stanton Hales Jr., Project Officer
Barnegat Bay Partnership — date

Approved by: _____ Oct. 21, 2016
Dr. Daphne Munroe, Lead-Principal Investigator
Rutgers University — date

Approved by: _____ Oct 21 2016
Dr. Jacob Goodwin, Co-Investigator
Rutgers University — date

Approved by: _____ Oct 25 2016
Dr. James Vasslides, Co-Investigator, QA Officer
Barnegat Bay Partnership — date

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Approved by: _____ _____
Donna Ringel, Quality Assurance Officer — date
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Barbara Spinweber, Project Officer — date
U.S. EPA Region 2

Approved by: _____ 5/24/16
Marc Ferko, Quality Assurance Officer — date
NJDEP Office of Quality Assurance

Approved by: _____ 5/24/16
Dorina Frizzera, Project Officer — date
NJDEP Division of Science, Research, and Environmental Health

Approved by: _____ _____
Dr. L. Stanton Hales Jr., Project Officer — date
Barnegat Bay Partnership

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Dr. Daphne Munroe, Lead-Principal Investigator — date
Rutgers University

Approved by: _____ _____
Dr. Jacob Goodwin, Co-Investigator — date
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Dr. James Vasslides, Co-Investigator, QA Officer — date
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Dr. L. Stanton Hales Jr., Project Officer date
Barnegat Bay Partnership

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Dr. Daphne Munroe, Lead-Principal Investigator date
Rutgers University

Approved by: _____ _____
Dr. Jacob Goodwin, Co-Investigator date
Rutgers University

Approved by: _____ _____
Dr. James Vasslides, Co-Investigator, QA Officer date
Barnegat Bay Partnership

3.0 QAPP DISTRIBUTION LIST:

Signed copies of this Quality Assurance Project Plan (QAPP) and all subsequent revisions will be sent to the following individuals by electronic mail:

Barbara Spinweber, Project Officer, U.S. EPA, spinweber.barbara@epamail.epa.gov
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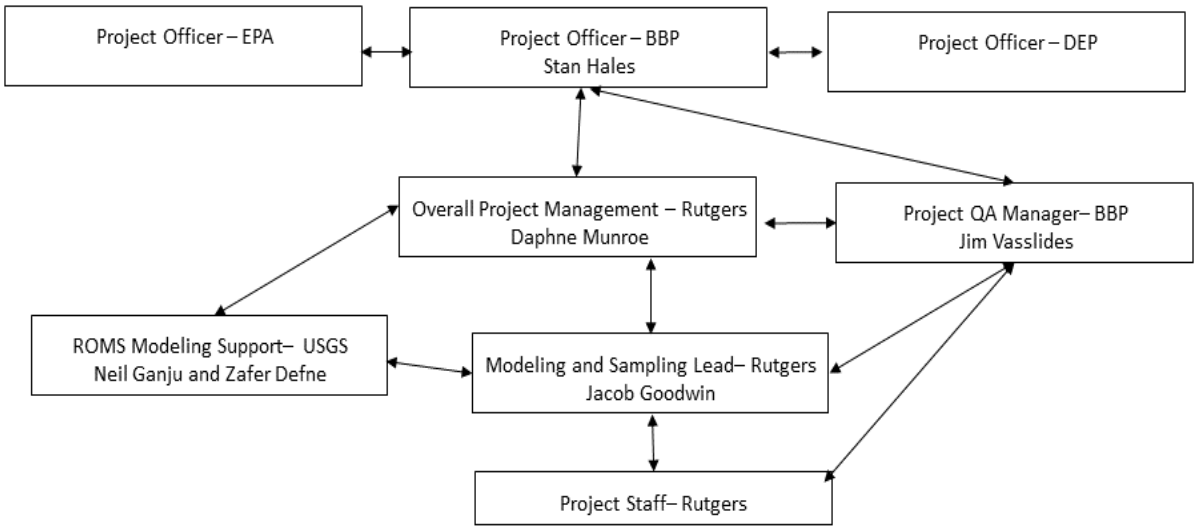
Dr. James Vasslides, Program Scientist, Barnegat Bay Partnership, jvasslides@ocean.edu
Dr. L. Stanton Hales, Jr., Quality Assurance Officer, Barnegat Bay Partnership, shales@ocean.edu

4.0 PROJECT TASK ORGANIZATION:

Overall project management and reporting will be the responsibility of Dr. Daphne Munroe. Dr. Jacob Goodwin will be responsible for model development, integration, and analysis, as well as overseeing larval field sampling and analysis. Neil Ganju and Zafer Defne of the USGS will be responsible for providing ROMS output files and assistance to Dr. Goodwin. The Rutgers staff will be responsible for assistance with sample collection and processing. Dr. Jim Vasslides will provide quality assurance management, reviewing data acquisition and data analysis protocols, and ensuring compliance with all elements of the QA Project Plan. Dr. Munroe will communicate with Dr. Stan Hales Jr., the Project Officer for the Barnegat Bay Partnership, who is responsible for ensuring the project is completed in accordance with the scope of work.

Organizational Chart-Lines of Communication



5.0 SPECIAL TRAINING/CERTIFICATIONS:

Training will be provided by Drs. Jacob Goodwin and Daphne Munroe to an undergraduate research assistant, Ms. Rachel Sheppard. Ms. Sheppard will be supported through an NSF REU program at Rutgers University. She will be trained on zooplankton sampling, sample management,

and processing techniques. Specifically, Rachel will be trained on FlowCam operation and procedures by members of the Munroe laboratory.

6.0 PROBLEM DEFINITION/BACKGROUND:

6.1 Problem Definition:

Success of restoration of wild shellfish populations requires both the survival of the restored or enhanced population, and the ability of that population to generate viable offspring (Pineda et al.,2007). Strategic planning of restoration efforts must therefore target locations that allow both survival of enhanced populations (adult shellfish) and support survival of larvae spawned from these populations. Hard clam larvae, like many other bivalve species, are released into the water column where they develop and disperse potentially long distances before returning to the bottom to settle and metamorphose into juvenile clams. The nature of the distances and direction they disperse relies on both physical dispersal (tides and currents) and larval behavior (swimming and growth) (Narváez et al., 2012a; Zhang et al., 2015). In estuarine systems like Barnegat Bay, the combination of physical forces and behavior can result in larvae moving among regions of the estuary and/or being lost from the estuary completely; the specific trajectories of dispersal will differ depending on where and when the larvae originate (Narváez et al., 2012a). These differences in larval dispersal among various release locations mean that some areas of the estuary will act as a better source of larvae, whilst other areas may generate larvae that are not well dispersed or lost from the estuary and therefore may not serve as good larval sources. Modeling can help to identify the possible patterns of dispersal and connectivity in the estuary and thus help in identifying areas that are better targets for both habitat and broodstock restoration efforts (North et al., 2008). Here, we propose to (1) use a coupled physical-biological *model to estimate connectivity* among candidate restoration sites and other potential hard clam habitat in Barnegat Bay, and (2) ground truth those estimates of connectivity with *larval sampling*. The connectivity patterns we will identify will provide important information with which strategic shellfish population enhancement and restoration planning decisions can be made. Thus this project fills in a key gap in our understanding of shellfish population dynamics, complements recommendations made by the BBP's Shellfish Working Group, and is a necessary precedent for successful restoration recommendations.

Goals:

Our goals for this project are fourfold. Our initial efforts will synthesize and integrate recent research in Barnegat Bay to generate model simulation conditions. Our primary efforts will use a coupled bio-physical modeling framework to estimate hard clam habitat connectivity patterns in Barnegat Bay. The larval dispersal pathways and connectivity estimates will provide important guidance for restoration strategies including broodstock and habitat enhancement. Our next goal is to ground truth those connectivity estimates with larval sampling in the bay. Samples of larval abundance and distribution will help further identify potentially realized connectivity patterns from the suite of modeled patterns already developed. Finally, we will create a documentary film that will be used to enhance public awareness about the importance of shellfish restoration and the scientific efforts that are underway in Barnegat Bay to rebuild clam populations in the Bay.

6.2 Background:

Shellfish provide a suite of benefits to coastal estuaries. They provide obvious economic and cultural value by supporting local fisheries, and provide ecological value through filter feeding. Increasingly, shellfish restoration and enhancement is being seen as a possible mechanism to help improve water quality in eutrophied estuaries (Carmichael et al., 2012; Rose et al., 2014). Hard clams (*Mercenaria mercenaria*) have long been part of the social, economic, and cultural fabric of Barnegat Bay (Kennish et al., 1984), with peak landings in the fishery for this species around the beginning of the 20th century (Ford, 1997). Today, landings are much lower and clams are increasingly being produced on aquaculture farms (Bricelj et al., 2012). The lack of continuous survey records of clam abundance and distribution of hard clams in Barnegat Bay make inferences about possible trends over time difficult (Bricelj et al., 2012). However, a recent hard clam survey performed by the NJ Department of Environmental Protection (NJDEP) Bureau of Shellfisheries has provided an estimate of Barnegat Bay hard clam populations that can be quantitatively compared to a prior estimate made in the mid-1980's (Dacanay, 2015). In 2012, the hard clam stock declined by 24% relative to where it was in the mid-1980's (Dacanay, 2015). Hard clam landings peaked at the turn of the century (Ford, 1997), and have been declining in recent decades (Bricelj et al., 2012), suggesting that today's clam population in Barnegat Bay is low relative to historical levels. For that reason, and because shellfish are critical ecosystem components, the Barnegat Bay Partnership has set restoration of shellfish beds as a key strategic priority in their 2012 – 2016 Vision for the Barnegat Bay Ecosystem.

7.0 PROJECT DESCRIPTION:

This project will use recently (2010-2015) collected hydrodynamic, chemical, physical, and biological data for Barnegat Bay to develop a model of larval hard clam dispersion that can be used to provide information about (1) optimal locations to site hard clam habitat/broodstock enhancement efforts that would improve the probability of these efforts seeding clams to other parts of the Bay, and (2) priority locations for habitat enhancement to increase overall clam habitat in the Bay. The physical circulation model will take advantage of an already established and verified hydrodynamic model for Barnegat Bay developed by USGS (Defne and Ganju, 2014). The model contains an operational particle tracking component, the ROMS-LTRANS submodel, which can be used to track particle dispersal among regions of the Bay. To provide connectivity patterns representative of hard clam dispersal, relevant particle behavior will be input for LTRANS based on an extensive body of literature documenting the growth, survival, feeding, and swimming of hard clam larvae. The LTRANS model will then be used to simulate larval dispersal patterns for larval releases starting in May through September (Carriker, 1961), and for larval durations of 14 days.

Coupled hydrodynamic models have been shown to be effective ways to make predictions of dispersal patterns for bivalve larvae. Unfortunately, less often have these predicted patterns been verified through field sampling. In the summer of 2016, we will sample bivalve larvae at fixed stations in Barnegat Bay to groundtruth predictions made using the larval model. If for any reason a planned station location is not accessible, samples will continue at the closest possible location to that station. The dispersal model groundtruthing effort will further enhance the value of the

dispersal model predictions to informing successful shellfish restoration efforts within Barnegat Bay.

Efforts to restore shellfish populations in Barnegat Bay are critical to coastal ecological health and sustainability; however, many in the general public are unaware of these efforts, or their importance. Our outreach program will develop a short (~6 to 8 minute) video documentary targeted at the general public that informs them about the importance of shellfish in coastal ecosystems, and how cutting edge technology such as larval modeling and image-detection can help refine and improve restoration efforts. The film, once completed, will be posted on the BBP YouTube Channel, which will be linked to both the BBP (bbp.ocean.edu) and Haskin Shellfish Research Laboratory (hsrl.rutgers.edu) websites. Advertisement for the film will occur by using banner planes flying at beaches along the Barnegat Bay coastline during the tourist season in the summer following the completion of the film. These tourists are a vast target audience who will have a familiarity and interest in the Bay. We believe banner planes are an effective way to have the public view advertised websites. The film will also be entered in film festivals (e.g., Beneath the Waves Film Festival), further increasing its impact and viewership.

Project Tasks and Schedule

	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan
Project Team Meeting	X			X						X		
QAPP preparation & approval	X	X	X	X	X							
Larval Model Lit Review	X	X	X	X								
Larval Dispersal Simulations		X	X	X	X	X						
Model Output Review & Field Sample Planning			X	X	X	X	X					
Field Larval Sampling					X	X	X	X				
Larval Sample Processing & Data Analysis								X	X	X	X	
Documentary Student Filming			X			X	X	X				
Documentary Student Film Editing									X	X	X	
Final Reporting, Manuscript Writing										X	X	X
AERS Conference Presentation	Will occur in the following spring (2017) once results are fully analyzed.											
Banner Plane & Film Showings	This will occur in the following summer (2017) once the film is finalized.											

8.0 QUALITY OBJECTIVES AND CRITERIA FOR MEASUREMENT DATA:

This project will apply literature derived values for key hard clam larval dispersal parameters (growth, swimming, feeding, etc) to a particle transport sub-model within an already established and verified USGS hydrodynamic model (Defne and Ganju, 2014). The quality objectives for these parameters is discussed in Section 9.0 Non-Direct Measurement. Field sampling for hard clam

larvae will occur during the summer of 2016 to ground truth predictions made using the larval model.

8.1 Precision:

8.1.1 Larval Hard Clam Sampling:

Hard clam sampling throughout the duration of the project will use the same collection devices and techniques in order to maintain precision of 200 L^{-1} of water collected for each sampling depth interval at each site. A FlowCam (model VS) sample analysis will be conducted onboard the vessel at each station to ensure the presence of bivalve larvae.

Temperature and salinity will be measured at each station because it is important for identification purposes (later described and also in Goodwin et al. 2014). A YSI 6600 datasonde will be used to measure temperature and salinity at the surface and bottom of each station where larval collections are made. Duplicate readings will be made at beginning and end of each day to assess if the unit is functioning correctly. Readings will need to be within 10% of each other to be considered acceptable. We anticipate making approximately 10 readings over the course of a field day, therefore the unit will not need calibration within a day.

8.1.2 Larval Dispersal Modelling:

Precision of model runs will be determined by using the same modeling software on the same computer for all model runs in all scenarios. For each model run, the physics will remain unchanged and thus will fully agree. The only inputs that will change among the runs of interest here, are the simulated biology of the particles being tracked. In addition, the model has specific 'alert flags' that stop runs in the case of improper input data. Furthermore, a report in the netCDF output files of the model reveal the time, area of release of particles, ending locations, and other important parameters that will help determine that the model run was successful.

8.2 Bias:

8.2.1 Larval Hard Clam Sampling:

Sampling hard clam larvae, and temperature and salinity, will be done over one season and therefore not reflect the seasonality and interannual variability of clam larvae in Barnegat Bay. However, we are purposely sampling during historically peak periods of larval abundance to validate our model predictions, and larvae will be sampled similarly throughout all stations reflecting the larval population for the specific time period sampled.

Bias in water quality measurements by the datasondes will be quantified through pre-deployment calibrations according to YSI specifications. The QC checks should not require more than slight adjustments to bring the instrument into agreement. Failed calibration checks will initiate a thorough inspection of the unit for obvious sign of malfunction (e.g., loose connections, damaged probes, power source, etc.). After any maintenance to correct problems, the unit will be re-calibrated with documentation on the appropriate field data form. If the unit will calibrate within the guidelines, water column measurements can be continued. If one or more parameters remain suspect, the nature of the problem will be fully documented on the field form, and the situation will

be reported to the Project QA Manager for resolution. If this situation occurs a backup instrument will be made available.

8.2.2 Larval Dispersal Modelling:
There is no bias associated with the modeling software itself, but bias could be introduced through the selection of the parameter values. Criteria for inclusion in the model are discussed in Section 9.

8.3 Representativeness:

8.3.1 Larval Hard Clam Sampling:

The goal of the larval clam sampling is not to determine the distribution of hard clam larvae throughout Barnegat Bay, but rather to validate the model by sampling in areas where larvae are predicted to occur and to evaluate relative numbers of larvae among stations. Most model observations predict larvae in all parts of the Bay despite a larger population in the south. However, it is important to cover the largest representative area with the resources available. Although preliminary model predictions show larvae disperse throughout the Bay, the lack of substantive adults in the northern portions suggest these areas may be dispersal limited, thus the sampling strategy covers the widest area to allow us to assess this possibility. The number and location of samples as described in Section 10 is sufficient to capture the predictive capability of the model.

8.3.2 Larval Dispersal Modelling:

The hydrodynamic model (ROMS) underpinning the larval dispersal model (LTRANS) has been shown to be highly representative of Barnegat Bay from a spatial perspective, and accurately reflects conditions in 2012, the year in which it was validated. Simulating the 2012 period is suitable because Superstorm Sandy appears to have minimally modified the hydrodynamics within the estuary. The water level response in the bay to Superstorm Sandy and tides remained unchanged after 2012 (Aretxabaleta et. al., 2014); preliminary simulations show expected bathymetric changes to be relatively small (excluding inlets and flood tidal shoals. Nonetheless, Defne & Ganju (2014) suggest that the residual circulation is mainly driven by tides and friction within the inlets under normal conditions. Therefore, one can anticipate the change in hydrodynamic circulation to be minimal as the inlet geometries have not changed substantially since 2012 (Defne pers. comm.).

8.4 Comparability:

8.4.1 Larval Hard Clam Sampling:

Larvae will be collected from two depth intervals (surface and bottom) on four different cruises to 10 stations in the Bay on each cruise. Data (e.g. larval samples) from each of the four cruises will be compared to evaluate the presence of larvae among the station locations, and over the period of the survey. Larval bivalve densities (#/L) are a standardized and common metric of abundance and can be compared to values from other studies and systems if needed.

8.4.2 Larval Dispersal Modelling:

Predicted dispersal patterns of clam larvae modeled will be compared with field observations over several model runs.

8.5 Completeness:

8.5.1 Larval Hard Clam Sampling:

Optimally, samples from two depths (surface and bottom) at each of 10 stations will be collected on each cruise day, for a total of 80 samples collected over the four sample days. The total amount (n=80) samples is a maximum goal, although the model may be validated with as few as 30 samples.

8.5.2 Larval Dispersal Modelling:

Model runs will implement larval releases (start times) that span May through September, and will include release locations throughout the Bay. This will create a simulation space that encompasses the range of realistic scenarios that could be observed in the field program. This simulated 'state-space' can then be validated by positions of field-collected larvae.

8.6 Sensitivity:

8.6.1 Larval Hard Clam Sampling:

FlowCAM ™ is an automated plankton imaging and sorting microscope. It is able to automatically process raw seawater samples by capturing images of each distinct particle (particle size ranges are variable, and will be set in this case for bivalve larvae 60-300µm) within the sample, then analyzing characteristics (size, color, etc) of those particles for sorting. Previous imaging of high density hatchery cultures of bivalves has shown that FlowCam can image >15 bivalve larvae per frame, and the frame rate is set such that a continuous record is made of the sample passing through the machine. Field samples, however, are not anticipated to be as dense as artificially reared hatchery cultures. FlowCam is a powerful tool for analysis of zooplankton samples that otherwise require slow and laborious handsorting. This tool can be used to rapidly process many more samples than would be possible otherwise and represents an opportunity for real time monitoring of clam larval densities. Sample processing with this tool not only allows rapid, automatic detection and analysis of larvae, it creates a permanent record of the images captured that can be used for subsequent reanalysis if necessary. FlowCam will be able to determine whether or not bivalves are present in live plankton samples *in-situ*. Later analysis using polarized light and subsequent FlowCam analysis on preserved samples will be used distinguish the hard clam larvae from other species, and to cross-validate the field techniques.

Temperature and salinity are both measured on the YSI to the hundredths of a degree/psu, which is well within the range required for species confirmation.

8.6.2 Model Parameters:

The larval transport model (LTRANS) is based on a particle-tracking model and is designed to predict the movement of bivalve larvae based on advection, sub-grid scale turbulence, and larval swimming behavior. The model will be sensitive to sub-grid scale turbulence from 2012 conditions and larval swimming behavior input (based on literature values). The hydrodynamic model (ROMS) has 1 km resolution and therefore the predicted salinity, currents (m s^{-1}), and other hydrodynamic quantities are interpolated in both time and space to provide a fine-resolution velocity field for advecting the clam larvae across this resolution scale (see North et al. 2008).

9.0 NON-DIRECT MEASUREMENTS (SECONDARY DATA):

9.1 Data Sources

This project will rely on existing data for the modelling component, including a previously developed and verified hydrodynamic model for Barnegat Bay and larval hard clam behavioral parameters. The Barnegat Bay ROMS hydrodynamic model will be provided by the developers (Defne and Ganju 2014), who are members of the project team. Values for the larval behavior parameters (e.g. swimming speed, vertical swimming behavior/timing, larval duration) will be obtained from existing published reports and primary literature. All data sources will be fully referenced, including links to databases, and any documentation of data quality.

9.2 Hierarchy of Data Sources

The hierarchy for hard clam larval behavior data will be as follows:

Data collected on hard clams from *within* Barnegat Bay
Data collected on hard clams *outside* of Barnegat Bay
Data collected on hard clams in a laboratory setting
Data collected on similar species from *within* Barnegat Bay
Data collected on similar species *outside* of Barnegat Bay
Data collected on similar species in a laboratory setting

9.3 Rationale for Selecting Data Sources

Due to the specificity of this project's data needs (*i.e.*, growth, survival, feeding, and swimming of hard clam larvae), in many cases there may only be a single data source available. If more than one data source is available, we will use the data with the highest pedigree as determined using the ranking in Section 9.2 above.

9.4 Secondary Data Quality Requirements

Data should meet the following quality requirements. If data do not meet the requirements, they may still be valuable for our purposes. If used, any shortcomings will be noted.

- Data were generated by a reliable source. Although the identity of the data generator does not guarantee data quality, it provides a simple screening criterion when multiple data sources are available. The following are indicators of data source reliability.
- Data generator has specific Standard Operating Procedures for data acceptance criteria and data usability (federal, state, and local agencies, or research institutions).
- Data are published in peer-reviewed articles or publications.

- Data have been collected for purposes similar to this research; *i.e.*, to assess hard clam larval behavior.
- Research Program has a QAPP or similar plan documenting quality assurance and quality control procedures to ensure data accuracy, precision, representativeness, and comparability.

- Data have been widely used and/or trusted by scientists and professionals in the subject.

Data taken from Barnegat Bay Comprehensive Action Plan projects are collected under NJDEP approved QAPPs and the researchers have certification and or approval by NJDEP to perform analysis in the field/laboratory. These data will have highest priority.

9.4 Secondary Data Review and Evaluation

The quality of the data used to populate the model will be based on data quality requirements defined in Section 9.4 of this document. In determining data quality, the completeness of the dataset will be assessed first, by inspecting the data description (usually metadata) or the dataset itself (whichever is more easily available). If completeness is deemed adequate, other quality requirements will be assessed by inspecting the QAPP, other QA/QC documentation, metadata, and/or other information obtained from data providers. Any concerns or deviations from the quality requirements will be noted and maintained with the dataset per the records management standards (Section 14).

10.0 FIELD MONITORING REQUIREMENTS:

10.1 Monitoring Process Design:

In the summer of 2016, we will sample bivalve larvae at fixed stations in Barnegat Bay to groundtruth predictions made using the larval model. The model connectivity generated for 2012 will be compared generally to the 2016 observations, with the recognition that these individual years may vary. Specific sample locations will be decided based on both consultation with BBP's Shellfish Working Group, and on model connectivity results, to allow sites to be informative towards viable restoration strategies and model groundtruthing. Larval samples will be collected during 5 sampling days following the likely spawning period. Specifically, two sampling days will be needed to cover the 10 stations over the entire area. These two sampling days will be conducted consecutively. Then after 7-10 days, the same sampling sites will be revisited on two more back-to-back days. The 5th day is reserved in case the previous four days are canceled. Hard clams are known to spawn over a protracted range mid-May through September; therefore, local industry knowledge and recent fecundity surveys will help inform specific timing for sampling.

Samples collected from the field will be preserved in buffered (Sodium Borate) 70% Ethanol and taken to the Haskins Shellfish Research Laboratory (HSRL) for analysis and archiving.

10.2 Monitoring Methods:

Samples will be collected by boat from two depths (surface and bottom) at 10 stations on each of 4 cruises (n=80), using a pump to collect water (200 L per sample) that is then sieved through a plankton net and cod end to retain particles between 64– 350 µm (see appendix 1). The intake rate for the hose needs to exceed 1.3-1.6 mm/second as that is the swimming speed of faster *M. mercenaria* larvae (Mileikovsky 1973, Kraeuter and Castagna 2001). To meet that criteria the system used will follow Goodwin (2015), which captured bivalve larvae in the Chesapeake

Bay. Retained particles from a second smaller (100 L) sample will be analyzed in real-time (no preservative) using automated image-detection, Flowcam™ (Álvarez et al., 2011). The full 200L sample will be sieved and rinsed with seawater filtered to 1um, into 100 ml containers that have 70ml of 100% buffered ethanol, then topped up with filtered seawater to 100mL (thereby preserving in 70% buffered ethanol). These preserved samples will be retained for subsequent analyses. Buffered ethanol will be used as a fixative because it will not degrade the delicate larval shells. Samples will be returned to the laboratory and available for additional analysis using Flowcam. In addition, polarized light birefringence patterns of *M. mercenaria* will be determined using hatchery reared specimens from the Barnegat Bay region. These patterns will be used to manually identify the presence/absence of *M. mercenaria* larvae in each of the 200L samples using the same polarized light technique. This is dependent upon other species in the water column but the reference samples from the hatchery will be reared in similar conditions to field collected specimen as suggested by Goodwin et al. (2014). Sample size (200 L) is sufficient for these multiple subsamples to be analyzed (Álvarez et al., 2011).

10.3 Field Quality Control (QC):

The volume pumped (200 L) will be monitored by pumping into large plastic containers that have been precalibrated with volume marks. Once the water reaches a pre-measured mark (200 L), the sample will be complete. If problems occur, such as lost, contaminated, mislabeled or improperly handled samples, these problems will be documented in the appropriate project record-keeping location (field or laboratory logs). If practical, replacement samples will be obtained. If differences between temporal or spatial characteristics of the replacement samples and the original samples have a bearing on calculations, modeling or other project activities or objectives, such differences will be noted in all subsequent documentation and products in which the replacement samples were used.

11.0 ANALYTICAL REQUIREMENTS:

11.1 Analytical Methods:

A second smaller sub-samples will be analyzed live (not preserved) onboard ship using FlowCam, an automated plankton imaging and sorting microscope. The live sample will be sieved through a plankton net and cod end to retain particles between 64– 350 μm (see appendix 1). This concentrated sieved sample will be diluted up to 10mL using filtered (to 1um) seawater. From this 10mL concentrated sample, a 2mL subsample will be imaged with FlowCam. This sample is independent of the full 200L sample to be preserved and therefore will not be retained physically. The sample will be passed through a 300um flow cell, at a rate sufficient to image the entire sample (these rates are set on the machine based on syringe size and flow cell aperture). All settings will remain constant among samples and will be recorded as metadata for each imaging run. If any of the image runs fail, a second run will be made using the remaining 8mL of the concentrated live sample. The FlowCam is able to automatically processes raw seawater samples by capturing images of each distinct particle within the sample, then analyzing characteristics (size, color, etc) of those particles for sorting. It is a powerful tool for analysis of zooplankton samples that otherwise require slow and laborious handsorting. This tool can be used to rapidly process many more samples than would be possible otherwise and allows these samples to be analyzed live. Sample

processing with this tool not only allows rapid, automatic detection and analysis of larvae, it creates a permanent record of the images captured that can be used for subsequent reanalysis if necessary. Preserved samples will also be taken back to HSRL and processed again using automated image-detection (Flowcam) for validation of real-time analyses. A subset of these preserved samples will also be analyzed using polarized light birefringence patterns (Goodwin et al., 2014, Appendix 3). Birefringence patterns are patterns emitted by bivalve larval shells under a polarized light. The patterns are species specific and can be discerned under a microscope. Both of these larval analysis methods will first be validated and 'trained' using larval libraries, as possible, generated from hatchery reared hard clam larvae to ensure accurate detection. The hatchery reared library will be obtained by choosing reared larvae that were reared within 10 units of salinity and 3 degrees Celsius in accordance with Goodwin et al. 2014.

11.2 Model Description, Calibration and Validation:

11.2.1 Model Description:

The circulation model to be used for this study is the **R**egional **O**cean **M**odeling **S**ystem (ROMS; Haidvogel et al., 2000; Shchepetkin & McWilliams, 2005). ROMS solves the three-dimensional hydrostatic primitive equations in terrain-following vertical coordinates using split-explicit time stepping. Computational features of importance to our simulations include coupling with the **L**agrangian **TRANS**port (LTRANS; North et al., 2011) particle tracking model. ROMS has been developed for, and successfully applied to, a wide variety of marine applications, including many studies of coastal and estuarine circulation (e.g., Warner et al., 2005; Liu et al., 2009; Zhang et al., 2009). The existing Barnegat Bay ROMS model is described in greater detail in Defne and Ganju (2014).

11.2.2 Hard Clam Distribution Simulation:

Larval behavior, growth, and swimming are components of larval dispersal models that have been demonstrated to be important in determining dispersal distance, settlement success, and connectivity patterns (Xue et al., 2008; North et al., 2008; Tian et al., 2009; Kim et al., 2010; Narváez et al., 2012a,b; Zhang et al., 2015). Extensive literature exists documenting the growth, survival, feeding, and swimming of hard clam larvae. For example, hard clam larval swimming speeds are reported to range from 1.17 to 1.33 mm s^{-1} for (Turner and George, 1955). Pelagic larval duration for hard clam larvae in Barnegat Bay has been observed to be relatively short, only 8 days (Carriker, 1961); however, laboratory experiments have demonstrated that the larvae may live 12 to 30 days under varying temperature and salinity conditions (Loosanoff et al, 1951; Davis, 1958). Size at metamorphosis, under optimal temperature and salinity conditions ranges from 210 to 230 μm (Davis, 1958). A further literature review will be performed to gather additional hard clam larval behavior data for input into the LTRANS model. The addition of this information will improve the predictive ability of the model and provide greater confidence in using model outputs for planning purposes. Larval clam swimming and growth behavior will be modeled for each time step.

11.3 Analytical Quality Control:

If problems occur, such as lost, contaminated, mislabeled or improperly handled samples, these problems will be documented in the appropriate project record-keeping location (field or laboratory logs). If practical, replacement samples will be obtained. If differences between temporal or spatial characteristics of the replacement samples and the original samples have a bearing on calculations, modeling or other project activities or objectives, such differences will be noted in all subsequent documentation and products in which the replacement samples were used. Samples that are processed by FlowCAM will be imaged and QC checks will be made by the technician running the system. Secondarily, Drs. Munroe and Goodwin will view the images for additional QC. Because the images will be archived digitally, these QC checks will be done both on board the cruise and after field work is complete. All data entered from the laboratory and field will be re-checked by at least one other person (than the one that recorded it) for QC.

12.0 SAMPLE HANDLING AND CUSTODY REQUIREMENTS:

Prior to all sampling dates all materials will be cleaned and an inventory of materials necessary for the field and in the laboratory will be completed (Appendix 1.2).

12.1 Field Data:

12.1.1 Field Data Forms:

The project field crews will record their raw field data on hard copy data sheets. The field sheet will record site information (date, time, site, weather) as well as water quality data measured on site.

12.1.2 Site/Sample Identification:

Sample ID numbers, date of data collection, and data parameters will be marked on all sample containers (described previously). Sampling packets for each site will be prepared prior to the sampling date by placing a complete set of field data forms and pre-labeled sample vials into the appropriate dry box. Samples will be processed on-station using FlowCam and brought back preserved to the laboratory for processing in the laboratory according to the appropriate SOP standards (Appendix 2.2). Sample metadata and larval counts will be entered into a central database (excel) by the laboratory researcher. All data entered will be checked for accuracy of transcription from field sheets by a second technician.

12.2 Biological Samples:

When the field crew returns to the dock or staging area, they will double check the field samples and respective data forms to verify that all samples and supplies are accounted for by comparing actual sample containers against the field data forms (Appendix 1.4). In the event that a sample is missing, the person checking samples will record the sample as missing on the data form. The crew responsible for the collection of that sample will be informed so that they may check the sample storage areas on the vessel. It may be that conditions in the field prevented the collection of a particular sample; in that situation, the reasons should have been recorded as a comment on the field log. If the sample is not recovered, the crew chief will make the decision for corrective action, whether simply to re-sample while still in the area or to schedule a make-up sampling on a later

date. Once a complete set of field collected samples (n=12 per cruise) are received at the laboratory, a master list of all samples and associated metadata will be kept in the Munroe Laboratory.

13.0 TESTING, INSPECTION, MAINTENANCE, AND CALIBRATION REQUIREMENTS:

Appendix 1.2 shows a list of field equipment and instruments used in this project. Both field and laboratory equipment and instruments require routine calibration checks to verify that their performance is within acceptable quality standards. The following sections will discuss the procedures and frequency for the various instrument calibrations that are key components in the collection of accurate environmental data.

13.1 Instrument/Equipment Testing, Inspection and Maintenance:

When necessary, equipment that may be utilized to collect or analyze environmental data for long term sampling will have periodic maintenance and calibration verification performed by manufacturer's representatives or service consultants. These procedures will be documented by date and the signature of the person performing the inspection.

13.2 Instrument/Equipment Calibration and Frequency:

13.2.1 FlowCam Calibrations:

Efficiency estimates of sampling are performed on a per sample basis on FlowCam. In addition, a duplicate sample will be run every 10th sample to ensure machine consistency, and the flow cell will be cleaned with manufacturer recommended protein digestion cleansers hourly during continuous operation. During down time greater than 30 mins between sample runs, full cleaning of the flow cell will be performed and the cell held clean and dry awaiting subsequent sampling. Prior experience, in which 48 continuous hours of live sample analyses were run,, demonstrated that this is sufficient to ensure reliable continuous operation.

All other sampling gear and laboratory instrumentation will be calibrated (if possible) as per manufacturer's recommendations or common sense to ensure proper function. Other equipment such as freezers, fridges, ice machines and polarized light microscopes should be monitored on a routine basis during periods of use to ensure their performance.

13.2.1 Polarized light Calibrations:

Polarized light microscopes will be calibrated per manufacturers recommendations to ensure proper function. The technique will follow Goodwin et al. (2014) included as Appendix 3.

13.2.2 Y.S.I. Data Sonde Calibrations:

The 6600 series YSI data sonde will be calibrated and checked against known standards prior to each cruise in accordance with YSI protocols.

14.0 DATA MANAGEMENT:

This project requires that each data generating activity, both field measurements and laboratory analyses, be thoroughly documented. Data will be recorded in a variety of paper and digital formats. Specific formats for both written and electronically recorded data will be prescribed

to document the field monitoring and pertinent steps of laboratory analyses. Once the field crews have returned to the lab, all data sheets will be entered manually into Microsoft Excel. All data sheets used in the laboratory will also be recorded in Excel. All electronic data will be quality checked by a technician to correct any transcription errors. All data will be backed up on the HSRL servers.

A study file containing planning documents (QAPP), SOPs, field data sheets, laboratory notebooks or work sheets, study-related correspondence, records of peer reviews or QA assessments (reviews), and reports and publications will be maintained. These records will be archived by HSRL for at least 3 years after the conclusion of the project.

14.1 Field Activities:

Field crews will rely primarily upon hardcopy field data forms to record most field collected data. Standardized hardcopy forms will be used (Appendix 1.4). FlowCam contains a built-in computer with spreadsheet functionality. A running field data log will be kept on FlowCam harddrive, and the data contained therein, as well as the FlowCam image data collected will be backed up to a thumb drive regularly during cruises. The data recorded on field data sheets will be transcribed into an electronic spreadsheet within a reasonable time following collection (target period, two weeks). To ensure consistency, one person will be responsible for the data entry. Data entry will be straightforward and user friendly; the fields in the electronic format will closely resemble the hardcopy raw data forms. Hardcopy data forms filled out for a given station will be compiled, and the original field sheets will be archived, as well as backup disks for all electronic files. These raw data will be kept on file at HSRL for a minimum of 3 years.

A systematic approach of sample tracking will be used to ensure accountability for the handling, storage, and transfer or shipment of the field collected samples. Chain-of-custody documentation will include the following basic components:

Sample Collection:

- A master inventory of all field samples that are expected to be collected, with check off fields providing documentation of all samples that are collected, and a check off field for those received at the lab.
    - In the case of a discrepancy between the expected, collected and received sample, a note will be made on the master inventory explaining why (eg. Sample was lost due to breakage).

14.2 Laboratory Analyses:

As with field collected data, the overall flow of data generated from laboratory analyses will follow the route established below:

Data Generator (raw data from laboratory)
↓
Project Manager (initial validation and formatting)
↓
Quality Assurance Officer (additional validation/verification, and formatting)
↓

Upon issuing appropriate advance notification (i.e., minimum of 2 weeks), Barnegat Bay Partnership (BBP) and US EPA maintains the authority to access the active files and/or request copies of specific information at any time. In addition, the full set of data will be part of the study file which BBP and US EPA be able to access at the completion of the project.

Whenever changes or updates are made to the QA Project plan, copies of the most current copy will be electronically transmitted to all persons on the distribution list specified in the QAPP distribution list. This will be the responsibility of the Project Manager (Daphne Munroe).

15.0 ASSESSMENTS AND OVERSIGHT:

The following sections outline the structured data reviews and assessments of data quality planned for the project. Note: Routine audits will be conducted by the Quality Assurance Officer during the course of the project, and will include review of any project environmental data collection activity.

15.1 Field Monitoring:

15.1.1 Field Crew Authorization:

Field teams will be responsible for the collection of environmental data and samples from the sampling sites. SOPs and guidelines are provided to all team members to help ensure that the data collected are of known quality. These guidelines allow for the use of different equipment (e.g., work vessels, etc.) as long as the data generated meet acceptability criteria. Such performance-based QA/QC is a key factor to the project success in deriving comparable data from diverse participants.

15.1.2 Field Reviews:

Field reviews will be carried out at the beginning of the field sampling window by the Project QA Manager. Any minor deficiencies observed during field surveillance (e.g., slight deviation from approved procedures, labeling irregularities, data reporting, etc.) should be immediately pointed out to the crew and corrective actions imposed on-the-spot. The evaluator will document with a brief note on the checklist and no further write-ups are required. If significant deficiencies (i.e., data quality is seriously compromised) are observed, the evaluator will make the appropriate on-the-spot correction, and, if the case warrants, call a halt to the field activities until the problems are resolved to the satisfaction of the Project QA Manager. All cases of this nature will be documented through a written report submitted to the Project Manager. A completed checklist along with a copy of the completed field data forms, and sample checklist from each sampling date provides the basic documentation for an evaluation of the crew's overall performance on this sampling date. All field review data will be maintained as part of the study file (Section 14.0).

15.2 Laboratory Activities:

15.2.1 Laboratory Approval:

All samples will be processed at HSRL using the SOPs in Appendix 2.2. All instruments will be calibrated and maintained according to manufactures conditions and SOP standards (Section 13; Appendix 2.2).

Prior to the start of sample analysis, all laboratory personnel will be required to complete laboratory training for the FlowCam SOP to be authorized to processes samples collected from the field. Training will consist primarily of hands-on sessions during which laboratory analysis will be instructed by the Program Manager (and associates) on the SOP developed/adopted for the project. Training for all laboratory personnel will culminate with an approval exercise in which crew members are observed and evaluated as they perform the full suite of laboratory analysis. Individuals that successfully demonstrate technical competence and a thorough appreciation of laboratory QA/QC requirements will then be authorized to initiate lab activities. If an individual fails to qualify on some aspect, the individual will receive further instruction in the area of their deficiencies until they perform at an acceptable level. All polarized light analysis will be conducted by Dr. Goodwin who is already proficient in this task (e.g. Goodwin et al. 2014, Goodwin et al. 2016). Polarized light microscopy allows for bivalve (e.g. mussels oysters, clams, scallops) to be identified.

15.2.2 Laboratory Reviews:

Laboratory reviews will be carried out at the beginning of the analysis period by the Project QA Manager. Any minor deficiencies observed during sample processing (e.g., slight deviation from approved procedures, labeling irregularities, data reporting, etc.) should be immediately pointed out to the analysts and corrective actions imposed on-the-spot. The evaluator will document with a brief note on the checklist and no further write-ups are required. If significant deficiencies (i.e., data quality is seriously compromised) are observed, the evaluator will make the appropriate on-the-spot correction, and, if the case warrants, call a halt to the laboratory activities until the problems are resolved to the satisfaction of the Project QA Manager. All cases of this nature will be documented through a written report submitted to the Project Manager. A completed checklist along with a copy of the completed field data forms, and sample checklist from each sampling date provide the basic documentation for an evaluation of the crew's overall performance on this sampling date. All laboratory review data will be maintained as part of the study file (Section 14.0).

16.0 DATA REVIEW, VERIFICATION, VALIDATION AND USABILITY:

The data generated during the project will be systematically reviewed with varying levels of scrutiny at several junctures along the path from time of collection to final reporting; from quick, on-the-spot screening to in-depth evaluation against established criteria or standards. For much of the field collected data, the first level of validation, a cursory screening, will occur as data are recorded; persons conducting and documenting real-time observations should be aware of the range that constitutes realistic values for a specific measure. With other types of data, the initial validation may not occur in such an immediate time frame. Nonetheless, most data are amenable to some form of quick screening soon after being generated and the responsibility for this first- cut

validation falls on the personnel performing the measurement. In addition, most laboratory analyses of the project samples will be monitored by a series of in-stream QC checks that indicate the general level of data quality for a given batch of samples. In addition, documented verifications are required to determine if data quality remains at a level acceptable for the program. The following sections outline the format and procedures to be used for evaluating and documenting data quality for the project and discuss how issues will be resolved when they occur.

16.1 Data Review, Verification, and Validation:

16.1.1 Review of Field Data:

A first review of field data occurs as the data are being collected by the field crews (e.g., are these data in the ballpark?). If the field personnel encounter situations where they question the validity of data they are collecting, they should immediately refer back to the appropriate SOP, attempt to isolate and resolve the problem; if they are unable to do so, then they must describe the situation in writing on the appropriate data sheet, and, as soon as possible, consult with their respective senior Field Manager or Project Manager for corrective actions.

The next level of review takes place as the Project Manager double checks that samples were properly checked in to the lab and consolidates and formats the field data. Most of the field crew will use hardcopy data sheets to record the bulk of field data; therefore, the data must be transcribed into a computerized format. Data will be transcribed to electronic format upon return. All hard copy originals will be placed in a secure file; and the electronic copies will be backed up on the HSRL servers.

During the data entry process, the field data will be screened for missing or errant information based on instrument sensitivity. All field data will be subject to an evaluation of the relative frequency of transcription errors enacted going from hardcopy into the electronic format. To determine this, a randomly selected subset of at least 10% of the station packages (the entire set of field data sheets submitted for a given station) will be pulled and the data (primarily, measurements or numerical values) manually compared against the electronic version on a field - by-field basis. Any errors will be listed in the data logbook and a final tally derived for the station. The total number of transcription errors for a complete set of data sheets should not exceed 5. If more than 5 transcription errors are found, the entire set of field data sheets will be pulled and re-examined for review and check for errors. If corrective actions are initiated (e.g., correcting a spelling error on the copied data form), the correction must be legible and the person who made the correction must document the alteration with their initial and date.

16.1.2 Review of Laboratory Data:

All laboratory data generated for the project will be systematically reviewed and evaluated by both the Project Manager and the Project QA Officer. Upon receipt of a data set, a temporary file will be created and a series of error checks developed (checks will vary with SOP) will be performed to ensure the data: 1) are within specified ranges appropriate to each parameter measured, 2) contain all required fields, 3) have encoded valid values from constrained look-up lists where specified, and 4) are in the correct format (text in text fields and values in numeric fields, etc.).

A first review of laboratory generated data occurs as the data are being processed by the laboratory analysts (e.g., are these data in the ballpark?). If the analysts encounter situations where they question the validity of data they are collecting, they should immediately refer back to the appropriate SOP, attempt to isolate and resolve the problem; if they are unable to do so, then they must describe the situation in writing on the appropriate data sheet, and, as soon as possible, consult with their respective senior staff or Project Manager for corrective actions.

The next level of review takes place as the Project Manager double checks that samples were properly checked into the lab, stored, and checked out, that the proper number of QC samples was analyzed depending upon SOP, as well as consolidates and double checks all calculations and formats all laboratory data. Most of the laboratory analysts will use hardcopy data sheets to record the bulk of the laboratory data; therefore, the data must be transcribed into an Excel spreadsheet. All hard copy originals will then be placed in a secure file; and the electronic copies will be backed up on the HSRL server and then be used for entering the data.

During the data entry process, the laboratory data will be screened for missing or errant information based on instrument sensitivity. All laboratory data will be subject to an evaluation of the relative frequency of transcription errors enacted going from hardcopy into the electronic format. To determine this, a randomly selected subset of at least 10% of the station packages (the entire set of laboratory data sheets submitted for a given sampling date) will be pulled and the data manually compared against the electronic version on a parameter by parameter basis. Any errors will be listed in the data logbook and a final tally derived for the parameter. The total number of transcription errors for a complete set of data sheets should not exceed 5. If more than 5 transcription errors are found, the entire set of laboratory data sheets will be pulled and re-examined for review and check for errors. If corrective actions are initiated (e.g., correcting a spelling error on the copied data form), the correction must be legible and the person who made the correction must document the alteration with their initial and date.

16.2 RECONCILIATION WITH USER REQUIREMENTS:

All data collected as part of this project must meet the QAQC standards defined by this QAPP. Missing data will be tracked with the sample inventory both in the field and in the lab. If possible missing samples will be replaced with additional samples. When that is not possible the data will be noted as missing in all electronic datasheets. The main data outputs for this project will be: clam larval presence, clam larval abundance, and model output.

16.2.1 Clam larval presence and abundance

Clam larval presence will be verified by comparing specimen of larvae reared in similar conditions. The larger stages are easier to confirm and therefore presence of clam larvae (> 4 days) will be confirmed by an expert. If another species that spawns in the system has the same exact pattern (though this has not happened with species investigated to date) the presence and abundance data of *M. mercenaria* may be rejected pending DNA or Raman laser spectroscopy verification.

The scenarios of LTRANS from the ROMS output files has been scored in a skill assessment and ranged from very good to excellent in predicting physical factors that influence BBLEH (Defne and Ganju 2014). The number of particles will be released in model runs will be consistent (i.e. statistically relevant) with previous peer reviewed publications (e.g. North et al. 2008, Defne and Ganjue 2014).

17.0 REPORTING, DOCUMENTS AND RECORDS:

All project documents and records will make use of a document notation system located in the footer region of all data sheets/reports. All documents will list the Project Name, Document Name and Revision number, Date, page number, and total number of pages in the document. All field and laboratory data will be maintained in hard copy in the data logbook, and electronic versions of the data will be maintained by the Project Manager. All data (hard and electronic copies) will be maintained at HSRL for a minimum of 3 years after the completion of the project.

Sampling collection and handling records will be maintained by the Project Manager and checked by the QA officer after each sampling date. Analytical logbooks will be updated following the processing of each sample by the analyst and audited by the QA officer. All QC sample records and equipment calibration records will be maintained as part of the appropriate analytical logbook and will be observed with the logbooks. The project manager will prepare progress reports and submit them to the Barnegat Bay Partnership, as required by the contract between BBP and HSRL. Data verification, analysis, completion of the modeling scenarios, and associated recommendations will be included in the final report to the Barnegat Bay Partnership which will be submitted in 2017.

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FIGURES

Figure 1. Map of Barnegat Bay watershed.

APENDECIES

Appendix 1: Field sampling Data Sheets, Checklists, and SOPs

A1.1 Example Field Cruise Plan Sheet

Example Cruise Plan field efforts Clam Connectivity Survey

Cruise: CCS-01

Date: May 10, 2016

Vessel: *R/V*

Area of Operations: Barnegat Bay

Scientific Personnel: Dr Daphne Munroe, Dr. Jacob Goodwin, Captain, research assistance (all personnel will be listed)

Objectives: The objectives of this cruise are to 1) determine the presence of hard clam (*Mercenaria mercenaria*) larvae in Barnegat Bay, NJ

Operations: There are 10 planned stations (5 stations per cruise). At each station, a plankton sampler will be deployed. Using a hose attached to a frame, water will be pumped from 2 depth intervals (bottom, surface) through a 44 µm mesh net to collect bivalve larvae. Samples will be concentrated and washed with seawater into 100 ml glass jars containing 70 ml of buffered (Sodium Borate) ethanol.

Station Locations: The direction of sampling will depend upon the weather and will be determined on the morning of each cruise. The following stations are planned:

NEED LIST OF STATIONS HERE

Contact Information: Jake Goodwin Cell Phone, Daphne Munroe Cell Phone, Captain, Crew:

A1.2 Example Field Cruise Supply sheet

To be signed off by Project Manager prior to cruise _____

Make sure that the following items are prepared and are on the boat or are ready in the laboratory once the sampling is complete. Check off each item as it is addressed.

pump (both diaphragm and priming pump) and hose
bucket rig
batteries
GPS (on vessel)
field laptop

FlowCam
FlowCam cleaning chemicals
nets
codends
Bucket for net
sieve (44 micron) - 2
squirt bottles for washing down codends - 2
Pre-labeled sample jars with preservative (20 per cruise)

tool box (duct tape, wire cutters, rope for tie-down, bungees, wire ties, velcro)
charts
pens, pencils, sharpies
label tape
waterproof field notebook (cruise log)
personal safety equipment

A1.3 Field Standard Operating Procedures

Once at the Station:

1) Obtain depth from shipboard echosounder and record depth
2) Record environmental variables at bottom and surface (temperature, salinity)
3) Obtain bottom water column sample first and record all information on field log sheet.
4) Take sub-sample for immediate (live) processing with FlowCam
5) Preserve and label (archive) remaining sample
6) Repeat 3 to 5 for surface water sample
7) Supervisor/delegated technician reviews all data is recorded on station
8) Team moves on to next station.

Upon return:

Field Sheets

1) Field sheets are reviewed to verify that all stations and samples are accounted by the project manager (initials on field sheet; Appendix 1.4).
2) Sheets are placed in designated folders/binders in an approved location at HSRL
3) Photocopied sheets are provided to dmunroe@hsrl.rutgers.edu

Bivalve larvae:

1) Larval samples are cross-checked with field sheets to assure all samples are accounted for. The supervisor or senior technician should initial on the inventory sheet that all samples are accounted for. If any samples are missing, the site should be revisited at the next possible opportunity to obtain a replacement sample.
2) All water samples are processed (or "fixed") within 0.5 hours of collection. All samples that were "fixed" will be analyzed (via FlowCam and/or cross-polarized light) within 10 weeks of sample collection.

A1.4 Example Field Cruise Data Sheet

Cruise	Date	Station	Depth (m)	Sample interval	Latitude	Longitude	Temperature °C	Salinity	Time pumped (s)	Larvae from FlowCam	Sample label	Shore sign in (initial)
CCS-01	5/15/16	1	7	bottom	-74.11225	39.856653	26	32	190	12	CCS-01-B	
CCS-01	5/15/16	1	0.5	surface	-74.11225	39.856653	27	30	185	214	CCS-01-S	
CCS-01	5/15/16	2	10	bottom	-74.2246	39.706653	26	32	190	0	CCS-02-B	
CCS-01	5/15/16	2	0.5	surface	-74.2246	39.706653	27	30	185	14	CCS-02-S	
CCS-01	5/15/16	3										
CCS-01	5/15/16	3										
CCS-01	5/15/16	4										
CCS-01	5/15/16	4										
CCS-01	5/15/16	5										
CCS-01	5/15/16	5										
CCS-01	5/15/16	6										
CCS-01	5/15/16	6										
CCS-01	5/15/16	7										
CCS-01	5/15/16	7										
CCS-01	5/15/16	8										
CCS-01	5/15/16	8										
CCS-01	5/15/16	9										
CCS-01	5/15/16	9										
CCS-01	5/15/16	10										
CCS-01	5/15/16	10										

Appendix 2 Laboratory Datasheets, Checklists, and SOPs

A2.1 Example Laboratory equipment

Gloves
FlowCam
FlowCam cleaning supplies and focusing standards
Pipettes
Sieve
Sedgewick Rafter slides
Polarized light microscope
Distilled water
200 mL beaker
Brass sieve series (15um – 2mm)
Sodium Borate buffer

Appendix 2.2 Example Laboratory standard operating procedures

FlowCam Processing

1. Laboratory Procedure for Sample Analysis with FlowCam:
    a. Prime and focus FlowCam using disposable standard.
    b. Initiate sample run and name file according to station and sample code name; include additional sample metadata in the notes prompt.
    c. Fully mix sample to homogenize and ensure a representative subsample.
    d. Remove subsample to be imaged with a clean pipette. Place ~1.5 mL into flow cell supply holder.
    e. Begin sample run. Image 1 mL. If no issues arise during the run, save the run.
    f. Double check the file by size sorting images and checking summary statistics (number of particles, size fractions of particles etc).
    g. Record summary run information and sample name on FlowCam sample processing summary spreadsheet.

Appendix 3 Goodwin et al. SOP for polarized light birefringence analysis

LIMNOLOGY
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Evaluating and improving a semi-automated image analysis technique for identifying bivalve larvae

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Abstract

Knowledge of the distribution, abundance, and transport of bivalve larvae is limited due to their small size, similar morphologies between species, and lack of an automated approach for identification. The objective of this research is to evaluate and improve the accuracy of ShellBi, a novel supervised image classification method that uses birefringence patterns on the shells of bivalve larvae under polarized light to identify species. The performance of the ShellBi method was tested by rearing *Crassostrea virginica* (eastern oyster) larvae at different temperatures (21.3 and 27.5°C) and salinities (10.3, 14.1, 14.4, and 20.5). Differences in rearing temperatures resulted in differences in classification accuracy, as did large variations in salinity (≥10 units). Classification accuracies increased from 67–88% to 97–99% when training sets included images of larvae reared in conditions similar to those of the larvae being classified. Additional tests indicate that misclassification rates ranged from 0 to 13% for false positives and from 0 to 22% for false negatives, depending on the proportion of oyster larvae in the sample. Results suggest that this technique could be applied to field samples with high accuracy as long as the images that are used to make classifications include larvae that were reared in conditions that are similar to those in situ. In addition, these findings demonstrate that the ShellBi method can be used to measure and identify bivalve larvae in a different system than the one for which it was developed, suggesting that the method has broad applicability in marine and estuarine systems.

Understanding dispersal pathways and connectivity is important for effective fisheries management strategies (Fogarty and Botsford 2007). The larval stage of bivalves is the least understood aspect of their life history, but it is important to understand because it is the stage during which dispersal takes place, which in turn, influences population connectivity and gene flow (Kennedy 1996; Pineda et al. 2007; Dame 2012; Munroe et al. 2012). Species identification is important for understanding dispersal and its effect on the population connectivity of bivalves because larvae of different species can exhibit variations in behavior that may result in large divergences in transport (Shanks and Brink 2005; North et al. 2008). However, studies of bivalve larvae are difficult to conduct because of identification challenges, small sizes of individuals, high mortality rates, and spatial patchiness (Boicourt 1988; Garland and Zimmer 2002).

Many identification techniques of bivalve larvae are too time consuming or expensive to apply when conducting sampling on a large scale. Accordingly, specific pros and cons of identification techniques of bivalve larvae are reviewed in Garland and Zimmer (2002), Hendriks et al. (2005), and Thompson et al. (2012a). Identification can involve time-consuming methods that rely on morphological differences (Loosanoff et al. 1966; Chanley and Andrews 1971; Lutz et al. 1982). More rapid molecular techniques include multiplex PCR (Hare et al. 2000), quantitative PCR (Wight et al. 2009), and fluorescent in situ hybridization with DNA probes (Henzler et al. 2010). Although quantitative PCR can provide some insight into the quantity of bivalve larvae, it does not provide information on the sizes of those larvae. Furthermore, these methods can have high costs and limitations on sample volume.

An alternative method for rapid identification is ShellBi. ShellBi can be an accurate, cost-effective, and rapid approach for identifying and measuring bivalve larval shells once the initial effort to prepare this technique for use in a new system is complete. ShellBi is a semi-automated image-processing approach that uses birefringence patterns on the shells of larvae that appear when subjected to polarized light (Tiwari and Gallager 2003a, 2003b; Thompson et al. 2012a). Under polarized light, color and texture-based features are extracted from digital images of the larval shells by pattern recognition software. The algorithm used in this work, a Support Vector

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Machine (SVM), generates decision boundaries that maximize differences between labeled categories (training images) and then applies the decision boundaries to classify new observations into those categories. For the ShellBi method, the categories are defined as groups of images of larval shells from known bivalve species (called 'training sets') and the observations are images of shells that need to be identified (called 'unknown sets'). In short, the classifier (the SVM) uses color- and texture-based features from the training set images to identify images of larval shells in the unknown set (Tiwari and Gallager 2003a, 2003b; Thompson et al. 2012a).

Thompson et al. (2012a) validated the ShellBi method with DNA and visual classification methods and improved it showing 98% identification accuracy for four hatchery-reared species *Argopecten irradians* (bay scallop), *Crassostrea virginica* (eastern oyster), *Mercenaria mercenaria* (quahog), and *Mya arenaria* (soft-shell clam). However, the species featured in their hatchery-reared training sets represented a simplified sample relative to field-caught larvae and larvae in situ may have had different growth rates due to environmental heterogeneities (Thompson et al. 2012a). Therefore, although obtained accuracies are high for identifying larvae reared in the hatchery, the effect of different growth conditions on shell formation between larvae reared in the hatchery and in the field may cause drops in accuracy. Therefore, improvements to the ShellBi method are needed when applied to field samples.

The overall objective of this research was to evaluate the use of the ShellBi method for identifying *C. virginica* bivalve larvae in the Choptank River, a tributary of Chesapeake Bay in Maryland, USA. Initially ShellBi was tested using bivalve species native to Cape Cod, Massachusetts, USA, and found in Waquoit Bay (Tiwari and Gallager 2003b; Thompson et al. 2012a). The bivalve species and physical characteristics of the mesohaline Choptank River differ from Waquoit Bay. Salinities near the surface of the Choptank River during the spawning season of oysters (May-October) are 0 to 14 and temperatures range from 17°C to 27°C (MDNR 2012). In contrast, Waquoit Bay water temperatures during May-October are 13°C to 26°C and salinities range from 28 to 32 (Thompson et al. 2012b). In addition to the overall objective of testing the ShellBi technique in a different system, the three specific objectives that guided this research were to 1) determine the influence of growth conditions on classification accuracy, 2) evaluate the influence of training set composition on classification accuracy, and 3) estimate misclassification rates of this method when applied to distinguish *C. virginica* larvae from other bivalve species found in the Choptank River.

Materials and procedures

Six bivalve species that are found in the Choptank River were spawned, their larvae were reared, and images of their shells were used to create training sets (Fig. 1). In addition, *C. virginica* larvae were reared in different growth conditions and imaged. A series of classification tests were conducted with the training sets and *C. virginica* images. Methods for spawning, rearing, imaging, and classifying larvae are described in this section.

Spawning and rearing bivalve larvae from the Choptank River

Six species of bivalve larvae were reared to obtain images for training sets: *C. virginica* (the target organism) and five other species that are abundant in the plankton along the mesohaline portion of Chesapeake Bay (Table 1). Adult specimens of the five species, *Ischadium recurvum* (hooked mussel), *Mulinia lateralis* (dwarf surf clam), *Mytilopsis leucophaeata* (dark false mussel), *Rangia cuneata* (Atlantic rangia), and *Tagelus plebeius* (razor clam) were collected from Choptank River field sites and brought to lab for spawning in 2009, 2010, 2011, and 2012. Some specimens of *M. lateralis* also were collected from the Corsica River (a tributary of Chesapeake Bay that is north of the Choptank River). Temperature fluctuation and strip spawning techniques were used to induce spawning (Chanley 1970; Kennedy et al. 1989). Larvae were raised at room temperature 23.0 ± 0.5°C (n = 30) (here and henceforth numbers after '±' are the standard deviation) and fed fresh *Isochrysis galbana* and *Thalassiosira pseodonana* (for D-stage and veliger larvae) and *Tetraselmis chui* (for pediveliger larvae). A subset of larvae was preserved in 80% ethanol buffered with sodium borate every two days from prodissoconch 1 through pediveliger stages so that different age/size classes for each species could be incorporated into training sets. The fixative was buffered to a target pH of 8.0 to inhibit dissolution of larval shells (Thompson unpubl. data).

In 2009, 2010, and 2011, multiple ages of *C. virginica* larvae (2-, 4-, 6-, 8-, 10-, 12-, 14- and 16-d old) were obtained from the Horn Point Oyster Hatchery where they had been reared at an average temperature of 25.9 ± 1.5°C (n = 30) and average salinity of 10.3 ± 0.9 (n = 30). These hatchery-reared *C. virginica* larvae were fed *Isochrysis galbana* and *Thalassiosira pseodonana* as D-stage larvae. For veliger stages, *Chaetoceros mulleri* was added. Pediveligers were fed *Tetraselmis chui* plus *Chaetoceros mulleri*. Algal concentrations averaged 5.7 × 10^4 cells mL^{-1} over the duration of the larval stages for hatchery-reared larvae. Larvae of *C. virginica* from 2009 were preserved in 80% ethanol buffered with sodium borate (Thompson et al. 2012a); larvae from 2010 and 2011 were preserved in 4% formalin buffered with sodium borate because larval shells stored in buffered ethanol began to crack after 2 years (Thompson and Goodwin unpubl. data). The preservative used to store larvae (formalin versus ethanol) did not interfere with the ability of ShellBi to classify bivalve larvae (Table 2).

In 2011, 1-d old D-stage *C. virginica* larvae were obtained from the hatchery and were reared at a mean temperature of 22.3 ± 0.4°C (n = 30) and mean salinity of 11.5 ± 0.3 (n = 30). Larvae were fed live cultures of *Isochrysis galbana* and *Thalassiosira pseodonana* (fed to D-stage and veliger larvae) and *Tetraselmis chui* (fed to pediveliger larvae) at an average concentration of 5.7 × 10^4 cells mL^{-1}. Subsets of larvae were preserved in 4% formalin buffered with sodium borate every 2 days up to day 20.

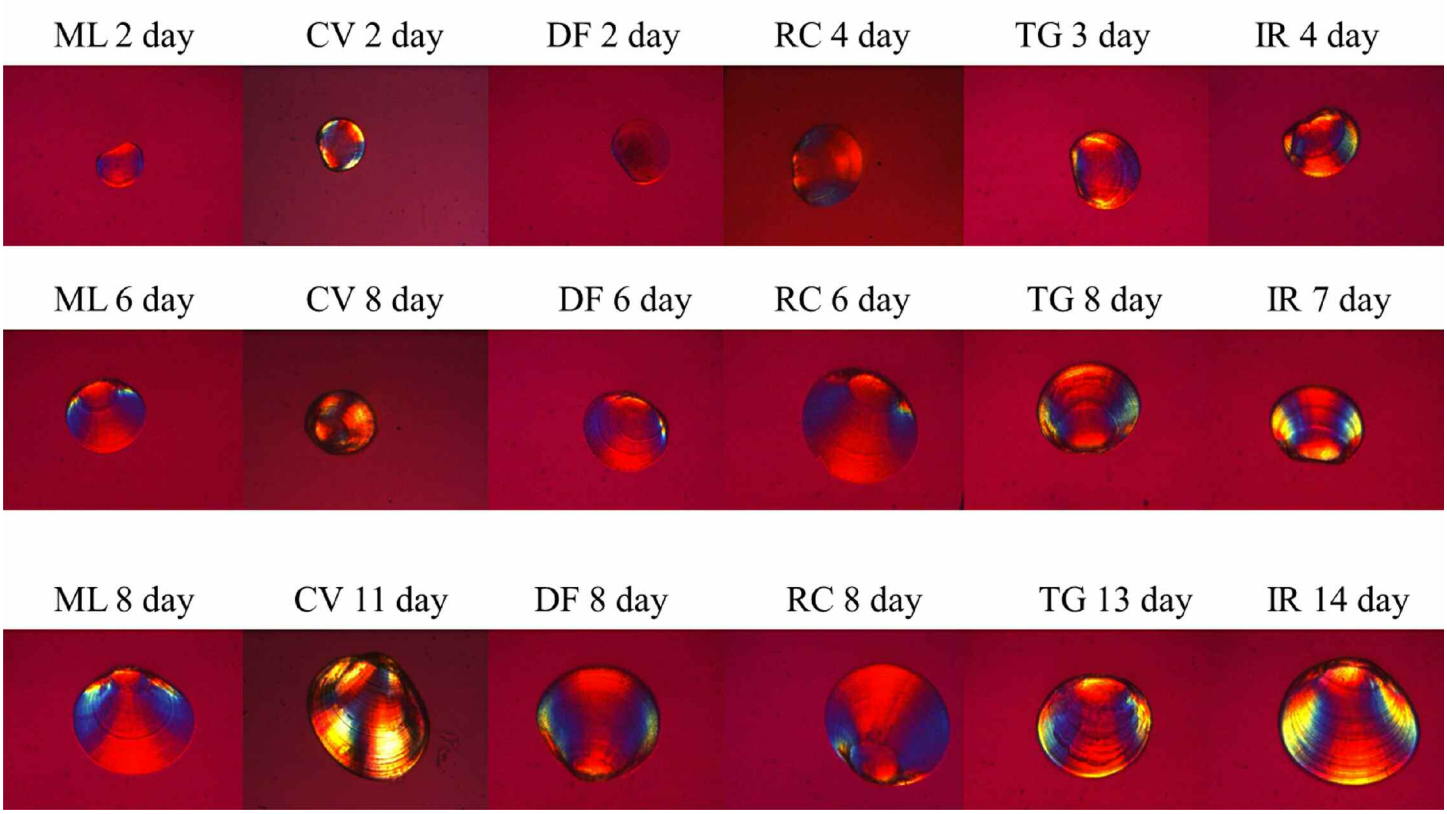

Fig. 1. Images under polarized light of the shells of six species of bivalve larvae used in the analysis ranging from early-stage veliger (top row, 2-4 d old) to late stage veliger (bottom row, 8-14 d old). Species pictured are *Mulinia lateralis* (ML), *Crassostrea virginica* (CV), *Mytilopsis leucophaeata* (DF), *Rangia cuneata* (RC), *Tagelus plebeius* (TG), and *Ischadium recurvum* (IR). Sizes of larvae range from 72-88 μm (top row), 95-155 μm (middle row), and 157-246 μm (bottom row).

Table 1. Spawning conditions for six species of bivalves that are found in the mesohaline region of the Choptank River.

Scientific name	Temperature	Salinity	Season
Ischadium recurvum	25-30°C	20	June-Nov
	(Chanley 1970)	(Chanley 1970)	(Chanley 1970)
Rangia cuneata	30°C	<15	late spring to early fall
	(Sundberg and Kennedy 1992)	(Sundberg and Kennedy 1992)	(Sundberg and Kennedy 1993)
Mytilopsis leucophaeata	30°C	0.5-18	Summer to fall
	(Kennedy 2011b)	(Kennedy 2011b)	(Kennedy 2011a)
Tagelus plebeius	30-32°C	10-30	June-Nov
	(Chanley and Castagna 1971)	(Chanley and Castagna 1971)	(Chanley and Castagna 1971)
Mulinia lateralis	28-30°C	20-30	May-Oct
	(Calabrese and Rhodes 1974)	(Calabrese and Rhodes 1974)	(Calabrese 1969)
Crassostrea virginica	28-30°C	12-27	Summer to fall
	(Kennedy 1996)	(Kennedy 1996)	(Kennedy 1996)

Rearing *C. virginica* larvae in different growth conditions

Larvae of *C. virginica* were reared at different temperatures, salinities, and food concentrations (parameters known to affect growth [Kennedy 1996]) to investigate how different growth conditions affect the classification accuracy of the ShellBi method.

Newly spawned *C. virginica* were obtained from Horn Point Oyster Hatchery and placed in 3-L glass rearing chambers within two temperature-controlled rooms. Water was collected from three sites within the Choptank River system (Tred Avon River, Harris Creek, and Choptank River at the Horn Point dock), and an external site (Chincoteague Bay) on the eastern shore of Maryland. Water was filtered to 1 μm in the field using a battery-operated pump (JABSCO model 50840-0012) and polypropylene cartridge system. Before rearing the larvae,

Table 2. Results of classification tests designed to determine if fixative type (ethanol versus formalin) influenced the classification accuracy of the ShellBi method. All fixatives for training sets and 'unknowns' were buffered with sodium borate. Training sets were composed of 250 images of the following species: *Crassostrea virginica*, *Ischadium recurvum*, *Mytilopsis leucophaeata*, and *Rangia cuneata.* Images of larvae in the training sets that were stored in either ethanol or formalin were used to classify images of *M. leucophaeata* that had been stored in either ethanol or formalin. Treatments denoted "ethanol & formalin" are composed of 100 images of *M. leucophaeata* that were stored in ethanol and 100 images of *M. leucophaeata* that were stored in formalin. The *M. leucophaeata* larvae were taken from the same cohort and stored in formalin or ethanol for an equal amount of time (11 months). All training sets had classification accuracies > 95%. Slightly lower accuracies were reported when training sets included images of shells stored in formalin (95% to 96%) compared with those stored in ethanol (97% to 98%). Based on the high classification accuracies for shells stored in both types of fixatives, it is concluded that the fixative used does not interfere with the ability of ShellBi to classify larvae.

Test Number	Fixative of training set	Fixative of unknown set	Percent classification accuracy
1	ethanol	ethanol	98.1
2	ethanol	formalin	95.2
3	ethanol	ethanol & formalin	97.3
4	formalin	ethanol	98.3
5	formalin	formalin	95.8
6	formalin	ethanol & formalin	96.1
7	ethanol & formalin	ethanol	97.1
8	ethanol & formalin	formalin	94.9
9	ethanol & formalin	ethanol & formalin	96.7

salinity was adjusted to provide a range of salinities that reflect conditions in situ in Chesapeake Bay. Salinity of the water collected at the Horn Point dock was raised to 10.3 and waters from the Tred Avon and Harris Creek were raised to 14.1 and 14.4, respectively, using Crystal Sea Marinemix (Marine Enterprises). The salinity of the Chincoteague Bay water was lowered to 20.5 using deionized (DI) water. Before starting this experiment, the water was filtered to 1 μm a second time.

The average water temperatures in the temperature-controlled rooms were 21.3 ± 1.0°C (n = 48) and 27.5 ± 0.6°C (n = 67). Each room contained 8 rearing chambers that held four salinity treatments (10.3 ± 0.7 [n = 58], 14.1 ± 0.7 [n = 63], 14.4 ± 0.6 [n = 53], and 20.5 ± 1.0 [n = 44]) using two chambers and two levels of food concentrations (high and low) within each salinity treatment. The concentration of algae fed to the larvae was based on the concentration of larvae in the containers (Helm et al. 2004), with low food treatments fed half the concentrations of the high food treatments. The ratio of larvae to algae in the high food treatments was on average 1:1.6 × 10^4, with the objective that the larvae would be fed to satiation. The average concentration of algae in the high and low food treatments were 9.2 × 10^4 cells mL^{-1} and 7.9 × 10^3 cells mL^{-1}, respectively. Algae were obtained from the Horn Point Oyster Hatchery and were composed of live cultures of *Isochrysis galbana* and *Thalassiosira pseodonana* (fed to D-stage and veliger larvae) and *Tetraselmis chui* (fed to pediveliger larvae). Subsets of larvae were preserved in 4% formalin buffered with sodium borate every 2, 4, 6, 8, 12, and 14 days in the warm chambers. In the cool conditions larvae took longer to develop to the pediveliger stage and were preserved every 2 days up to day 20.

Image acquisition for training and unknown sets

Images of all larval shells were taken by an Infinity 2.3C digital 8 megapixel camera mounted on a custom-built compound microscope fitted with a polarization filter and full wave compensation plate (λ). Larvae were first soaked in 40% bleach and 60% DI water buffered with sodium borate (hereafter referred to as buffered DI water) for a period of 15 min to remove tissue and break apart the valves of the shells. The larval shells were then sieved and rinsed with buffered DI water onto a Sedgewick Rafter slide. Digital images of individual shells were taken under 50× magnification at a resolution of 96 dpi. The microscope stage was moved manually or with a joystick attached to an automated stage to image one shell after another. Images were captured with shells at random orientations. A 12V 100W incandescent microscope bulb was used as a light source. Lumenera Analyze software (version 5.0.3 Lumenera Corporation) was used in conjunction with the digital camera to capture JPEG images. Settings on the software were adjusted so that they matched background color and cross polarization pattern as suggested in Thompson et al. (2012a) and kept constant between images. Major background color differences occurred throughout the day when a metal bracket was used for the full wave compensation plate which was near the light source of the microscope. Because these differences affected classification accuracies (results not shown), a plastic housing was used for the wave compensation plate to prevent background color drift.

To create a species category within a training set, 250 images of individual shells were selected for each species so that the images spanned the range of stages and sizes of the

larvae (prodissoconch-1 through pediveliger). Thompson et al. (2012a) found that at least 200 images should be used in a training set. Training sets were composed of different numbers of species. For example, a 6-species training set included 250 images of *C. virginica, I. recurvum*, *M. lateralis*, *M. leucophaeata*, *R. cuneata*, and *T. plebeius* for a total of 1500 images. All training sets were balanced: each species category had an equal quantity of images (250) with similar age representations of bivalve larvae.

Images of *C. virginica* shells from the experiment were used as unknown sets. The same imaging procedures that were used for the training sets were also used for *C. virginica* larvae reared in the growth experiment. There were 3288 images of larvae captured from the experiment. Those images were used to represent warm and cool conditions as well as four different salinity treatments.

Images were preprocessed before classification so that each larval shell, a region of interest (ROI), was defined and distinguished from its background (Thompson et al. 2012a) using MATLAB (version R2009a, Mathworks Inc.) and its image Processing Toolbox (version 6.3, Mathworks Inc.). The preprocessing (i.e., cropping) was performed using an automated ROI masking routine in MATLAB (Thompson et al. 2012a).

Image classification and analysis

Image classification was accomplished by extracting features from training sets, cross validating the training sets, extracting features from unknown images, and using the training features to classify unknown images (Thompson et al. 2012a). All images were processed using the Bivalve Larval Identification (BivLID) software implemented in MATLAB by C. Thompson based on algorithms used in Tiwari and Gallager (2003b) and Thompson et al. (2012a). Training set feature extraction and cross-validation were conducted before the classification of unknown images. The feature extraction process calculated 1104 Gabor texture features and 9 color-angle features for each image. A Principle Component Analysis (PCA) was then conducted using the Gabor texture features and color angles to isolate the 25 Gabor features that encompassed the most variability in the training set and to remove redundancy and noise (Zhao et al. 2010; Thompson et al. 2012a). After extracting and transforming features from the training set and unknown images, a Support Vector Machine (SVM) in BivLID was used for cross-validation and classification (Cawley 2000, http://theoval.cmp.uea.ac.uk/svm/toolbox/).

A leave-one-out cross validation procedure (LOO, Fukunaga and Hummels 1989) was run to assess performance of the training sets. This procedure left out one image from the training set, used features from the remaining images to classify the left-out image, and repeated this for all images to calculate cross validation accuracy for each category. Classification tests were also conducted. To classify an image, the SVM mapped the same features from the unknown image to the decision boundaries created with the training set using a one-to-one approach for each category (Lou et al. 2003). An "other" category was created so unknown images would not be classified as false positives, i.e., forced into a training set category to which they were not closely related (Davis et al. 2004). The output of the program indicates how many unknown images were classified into each training set category and the "other" category.

Larval shells were measured and statistical tests were performed to compare shell heights. To accomplish this, a script was created in MATLAB (version R2009b, Mathworks Inc.) to measure the maximum axis of a masked ROI of a larval shell as a measure of shell height. Nonparametric statistical tests were conducted because shell heights in all treatments were not normally distributed (Shapiro-Wilk, $\alpha = 0.05$, $P < 0.01$). Shell heights of *C. virginica* in the high and low food treatments were paired by salinity and temperature treatments for an even comparison (Sokal and Rohlf 1987). Median shell heights were not significantly different between larvae reared in high (95.9 μm, $n = 177$) and low (91.0 μm, $n = 177$) food treatments (Wilcoxon rank sum = 32750, Z = 1.39, $P < 0.17$, $n = 354$). Therefore images from high and low food treatments were pooled within each salinity and temperature treatment in further analyses. To determine if there was a difference in median shell heights between warm and cool treatments, a Wilcoxon rank sum test was employed with data pooled across salinity treatments. A Kruskal-Wallis one-way analysis of variance by ranks was used to test for differences in median shell heights between salinity treatments. After conducting the Kruskal-Wallis test, intergroup comparisons between salinity treatments were made using Mann-Whitley U tests. A Bonferroni adjustment was used to reduce type I error so that the P value for significance was set to 0.008 (Bland and Altman 1995). The number of larvae reared in warm and cool conditions was similarly represented across salinity treatments, and therefore, did not bias larval growth across salinity treatments for these tests. All statistical tests were performed using MATLAB (version R2012a, Mathworks Inc.).

Assessment

Tests were conducted to evaluate the influence of growth conditions on the classification accuracy of the ShellBi method, to determine the influence of training set composition on classification accuracy, and to estimate misclassification rates. A leave-one-out (Fukunaga and Hummels 1989) cross validation resulted in high cross validation classification accuracies (>90.8%) for all training sets except for a 6-species training set (74.7%) (Table 3).

The influence of growth conditions on classification accuracy

The effect of temperature on classification accuracy of a hatchery composed training set was tested using two training sets that contained *C. virginica* reared in warm conditions (a 3-species training set composed of 250 images each of *C. virginica*, *M. lateralis*, and *R. cuneata* and a 4-species training set

Table 3. Leave one-out (LOO) cross-validation accuracy of training sets for classifying *C. virginica*. The first column lists the analysis in which the training set was applied. The second column gives the two letter code of each species used in the training set (CV: *Crassostrea virginica*, RC: *Rangia cuneata*, ML: *Mulinia lateralis*, TG: *Tagelus plebeius*, IR: *Ischadium recurvum*, and DF: *Mytilopsis leucophaeata*). The third column lists the number of images in each training set. The fourth column gives the LOO percent accuracy for classifying *C. virginica.*

Analysis	Training set	Number of images	Percent cross validation accuracy
Temperature			
26.4 (hatchery 3-species)	CV, RC, ML	750	98.1
26.4 (hatchery 4-species)	CV, RC, ML, TG	1000	98.9
22.3 (cool Exp)	CV, RC, ML	750	97.1
Salinity			
10.3	CV, IR, RC	750	98.8
14.1	CV, IR, RC	750	99.6
14.3	CV, IR, RC	750	99.6
20.5	CV, IR, RC	750	99.2
Variation in growth conditions			
RC, ML, CV-2009	CV, RC, ML	750	99.6
RC, ML, CV-2009-2010	CV, RC, ML	750	98.5
RC, ML, CV-2009-2010-2011	CV, RC, ML	750	98.1
RC,ML,CV-2009-2010-2011-exp	CV, RC, ML	750	96.7
Larval stage			
Veliger	CV, ML, TG	750	99.0
D-stage	CV, ML, TG	750	96.4
Training set composition			
3-species	CV, RC, TG	750	95.6
3-species	CV, IR, RC	750	95.7
3-species	CV, ML, IR	750	92.4
3-species	CV, DF, RC	750	98.8
3-species	CV, DF, IR	750	95.6
3-species	CV, DF, TG	750	97.2
3-species	CV, IR, TG	750	94.8
3-species	CV, TG, ML	750	95.2
3-species	CV, ML, RC	750	98.1
4-species	CV, RC, TG, ML	1000	92.4
4-species	CV, RC, IR, ML	1000	92.4
4-species	CV, RC, IR, DF	1000	94.0
4-species	CV, DF, TG, IR	1000	94.0
4-species	CV, RC, DF, ML	1000	97.2
4-species	CV, DF, TG, ML	1000	94.0
4-species	CV, RC, TG, IR	1000	94.0
5-species	CV, RC, IR, TG, ML	1250	90.8
5-species	CV, RC, IR, TG, DF	1250	92.8
5-species	CV, RC, IR, ML, DF	1250	91.6
5-species	CV, RC, TG, ML, DF	1250	95.6
5-species	CV, IR, TG, ML, DF	1250	90.8
6-species	CV, RC, IR, TG, ML, DF	1500	74.7
6-species	CV*, RC, IR, TG, ML, DF	1500	92.1
3-category order-based (clams, oysters, mussels)	(CV), (IR), (RC, ML, DF,TG)	750	90.7
3-category order-based (clams, oysters, mussels)	(CV*), (IR), (RC, ML, DF,TG)	750	98.9

*Denotes that images of *C. virginica* larvae grown in different temperature and salinity treatments were added to the *C. virginica* training set category (Table 5).

that also included 250 images of *T. plebeius*). For both training sets, *C. virginica* larvae were reared in the hatchery at an average temperature of 25.9 ± 1.5°C (n = 30). The other species were reared in our laboratory at room temperature 23.0 ± 0.5°C (n = 30). The training sets contained images of larvae at similar age ranges (2-14 days old).

The 3- and 4-species training sets were used to conduct four classification tests in which the training sets remained the same and the "unknown" images of *C. virginica* shells from the experiment were varied. The two test sets were comprised of images of larvae reared in 1) the warm (27.5 ± 1.0°C, n = 67) treatment and 2) the cool (21.3 ± 1.0°C, n = 48) treatment. Each of these unknown sets included images of larval shells grown at all salinity levels and age ranges between 2-20 days old. The temperatures at which larvae were reared significantly influenced growth of the two treatments: larvae reared in cooler treatments had shorter median shell heights (77.0 μm, n = 365) than those reared in warm conditions (88.8 μm, n = 365) (Wilcoxon rank sum: 97903, Z = –12.7, P < 0.01, n = 730). The median shell height of larvae from the warm treatment was shorter, but not significantly, than the median shell height of the hatchery-reared *C. virginica* larvae in the training sets (114 μm, n = 916) (Wilcoxon rank sum: 107222, Z = -0.88, P = 0.39). On average, the accuracy of ShellBi for identifying *C. virginica* reared in the warm treatment was ~ 20% higher than the accuracy for identifying *C. virginica* reared in the cool treatment using 3-species and 4-species training sets (Fig. 2). In other words, the classification accuracy for *C. virginica* was highest when the temperature at which larvae in the unknown set were reared was similar to that of the training sets.

An additional analysis was conducted to test the effect of rearing temperature on classification accuracy using another training set composed of larvae reared in cool conditions. In this case, the training set was composed 250 images of each species reared in similar cool temperature conditions, *C. virginica* (22.3 ± 1.2°C, n = 58), and *Rangia cuneata* and *Mulinea lateralis* (23.0 ± 0.5°C, n = 30). This training set was used to classify *C. virginica* larvae from two treatments 1) warm (27.5°C, n = 1624) and 2) cool (21.3°C, n = 1664). The accuracy for identifying larvae from the cool treatment was 25% higher (91.0%) than the classification accuracy for larvae from the warm treatment (66.0%) (Fig. 2). Because shell heights differed between larvae grown in warm and cool conditions and because of the strong influence of temperature on classification accuracies, it is concluded that differences in temperature-dependent growth conditions between training sets and unknown sets influence the classification accuracy of the ShellBi method.

In addition to temperature, the effect of salinity on classification accuracy was tested using 3-species training sets composed of *C. virginica*, *R. cuneata*, and *I. recurvum*. The *C. virginica* used in the training sets and for the unknown sets were reared in the experiment at four salinities (10.3, 14.1, 14.4, and 20.5) and were pooled across temperatures. The images of *C. virginica* reared at the four salinities were used to create four different 3-species training sets. In addition to 250 images of *C. virginica*, each training set also had 250 images of *R. cuneata* and *I. recurvum* (reared in a salinity of 11.3). Each of the four training sets were then used to classify four unknown sets of 250 different *C. virginica* images from each of the three other salinity treatments. For example, the training set with *C. virginica* larvae raised in salinity of 10.3 was used to classify larvae from the three other treatments (14.1, 14.4, and 20.5). A total of 12 tests were conducted. High classification accuracies (>95%) occurred when training sets with larvae from low salinity treatments (10.3, 14.1, and 14.4) were used to identify "unknown" *C. virginica* larvae reared in the same low salinity treatments (Fig. 3). Accuracy dropped by 10% when these training sets were used to classify larvae raised in the higher salinity treatment (20.5) (Fig. 3). Training sets with larvae raised in the high salinity treatment (20.5) classified "unknown" larvae from the three lower salinity treatments with > 95% accuracy.

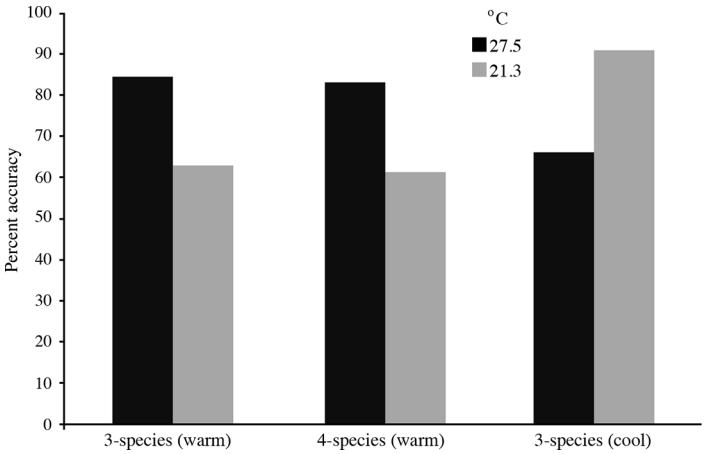

Fig. 2. Classification accuracy for *C. virginica* using two 3-species training sets (*C. virginica*, *M. lateralis*, and *R. cuneata)* and one 4-species training set (*C. virginica*, *M. lateralis*, *R. cuneata*, and *T. plebeius*). Images of shells of *C. virginica* were reared at 25.9°C for 'warm' training sets and at 23.3°C for the 'cool' training set. All three training sets were used to classify shells of *C. virginica* from warm (darker bars) and cool (lighter bars) treatments.

Median shell heights in treatments (n = 250 for each treatment) with salinities of 10.3, 14.1, 14.4, and 20.5 were 76.1 μm, 80.0 μm, 83.9 μm, and 98.3 μm, respectively. Shell heights were significantly different between the four treatments (Kruskal-Wallis test, df = 999, P < 0.01). Post-hoc pairwise comparisons were made using Mann-Whitney U tests. Salinity treatments were significantly different (P < 0.008, df = 499), except for salinity treatments 14.1 and 14.4 (P = 0.13, df = 499). Based on this and the results of the classification tests above, it is concluded that large (10 unit) differences in salinity-dependent growth conditions between training sets and unknown sets influence the classification accuracy of ShellBi.

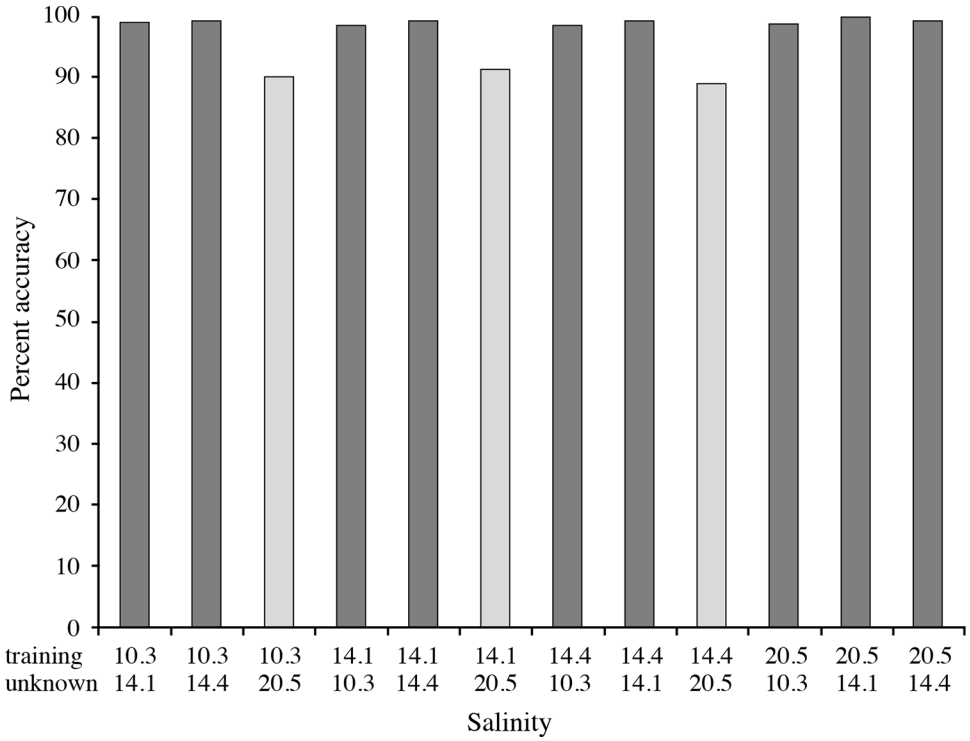

Fig. 3. Classification accuracies for shells of "unknown" *C. virginica* larvae raised in four different salinities (10.3, 14.1, 14.4, and 20.5) when classified with training sets composed of *R. cuneata*, *I. recurvum*, and *C. virginica* larvae, the latter of which were raised in the same four salinities. Numbers under each bar represent the salinity at which *C. virginica* were reared in the training set (upper number) and in the unknown set (lower number). Lighter bars indicate training sets in which larvae were reared at the lower three salinities and used to classify larvae in the high salinity treatment (20.5).

The influence of training set composition on classification accuracy

Three tests were conducted to determine if the composition of images in a training set influenced classification accuracy. (1) The first examined how changing the larval stage (D-stage versus veliger) within the *C. virginica* portion of the training set altered classification accuracy. (2) The second test was designed to identify how the number of categories in a training set influenced classification accuracy. (3) A third test was conducted to determine if increasing variation of growth conditions of larvae in the *C. virginica* portion of the training set affected classification accuracy.

(1) Larval images were broken down into 2 groups (1) D-stage larvae (comprised of larvae between 2-3 days old), and (2) veliger larvae (comprised of larvae between 6-20 days old). Two training sets composed of *C. virginica*, *M. lateralis*, and *T. plebeius* were created. All training sets contained the same images of *M. lateralis* and *T. plebeius*. Images in the *C. virginica* category were varied to form the two training sets that were comprised of (1) images of D-stage larvae raised in the hatchery and (2) images of veliger larvae raised in the hatchery. These training sets were used to classify unknown sets that were comprised of *C. virginica* images of (1) D-stage larvae from the hatchery, (2) D-stage larvae from the experiment, (3) veliger larvae from the hatchery, and (4) veliger larvae from the experiment. Results indicate that training sets containing images of D-stage *C. virginica* larvae classified "unknown" D-stage and "unknown" *C. virginica* veliger images with high accuracies (>98%). Training sets comprised of images of *C. virginica* veliger larvae and used to classify "unknown" D-stage *C. virginica* images had low accuracies (<29%). Based on these results, it is concluded that a training set should contain images of both D-stage and veliger larvae.

(2) Classification tests were conducted using training sets with various numbers of categories and the same set of unknown larvae. Images of *C. virginica*, *I. recurvum*, *T. plebeius*, *R. cuneata*, *M. lateralis*, and *M. leucophaeata* larvae were used to create nine 3-species training sets, seven 4-species training sets, five 5-species training sets, and one 6-species training set. These training sets were used to classify one unknown set comprised of *C. virginica* larvae from the warm and cool treatments of the experiment ($n = 998$). Results comparing the number of categories in a training set indicated that mean accuracies were 82% for 3-species categories ($n = 9$), 75% for 4-species categories ($n = 7$), 70% for 5-species categories ($n = 5$), and 67% for 6-species categories ($n = 1$) (Table!4). When the number of training set categories increased from 3 to 6, the accuracy of ShellBi dropped on average by 17% (Fig.!4). Within the 3-, 4-, and

Table 4. Percent classification accuracy of unknown *C. virginica* larvae from experiments (n = 3288) using training sets with different numbers and compositions of species. Training sets of 3-, 4-, 5-, and 6-species categories were comprised of *C. virginica* (CV), *R. cuneata* (RC), *T. plebeius* (TG), *I. recurvum* (IR), *M. lateralis* (ML), and/or *M. leucophaeata* (DF). 250 images were used for each category.

Training set	Percent classification accuracy	Number of images in training set
CV, RC, TG	69.5	750
CV, RC, IR	72.1	750
CV, ML, IR	82.1	750
CV, DF, RC	72.1	750
CV, DF, IR	99.8	750
CV, DF, TG	96.9	750
CV, IR, TG	97.1	750
CV, TG, ML	79.5	750
CV, ML, RC	71.8	750
CV, RC, TG, ML	66.7	1000
CV, RC, IR, ML	69.1	1000
CV, RC, IR, DF	72.2	1000
CV, DF, TG, IR	96.9	1000
CV, RC, DF, ML	69.2	1000
CV, DF, TG, ML	79.7	1000
CV, RC, TG, IR	69.5	1000
CV, RC, IR, TG, ML	66.6	1250
CV, RC, IR, TG, DF	69.6	1250
CV, RC, IR, ML, DF	69.3	1250
CV, RC, TG, ML, DF	66.8	1250
CV, IR, TG, ML, DF	79.6	1250
CV, RC, IR, TG, ML, DF	66.8	1500
CV*,RC, IR, TG, ML, DF	97.1	1500
order-based: (CV), (IR), (RC, TG, ML, DF)	87.8	750
order-based: (CV*), (IR), (RC, TG, ML, DF)	98.3	750

*Denotes that images of *C. virginica* larvae grown in different temperature and salinity treatments were added to the *C. virginica* training set category (Table 5).

5-species category training sets, classification accuracies varied by as much as 30% depending on which species combinations were used for each training set (Table 4). When the 6 species training set was grouped into a 3-category training set based on taxonomic order [1: Ostreoida, oysters (*C. virginica*), 2: Veneroida, clams (*M. lateralis*, *M. leucophaeata*, *R. cuneata*, *T. plebeius*), 3: Mytiloida, mussels (*I. recurvum*)], classification accuracy improved compared with the 6-species training set, from 66.8% to 87.8%. Therefore the number of categories in a training set and the species composition within them are important factors that affect the classification accuracy of *C. virginica* using the ShellBi approach.

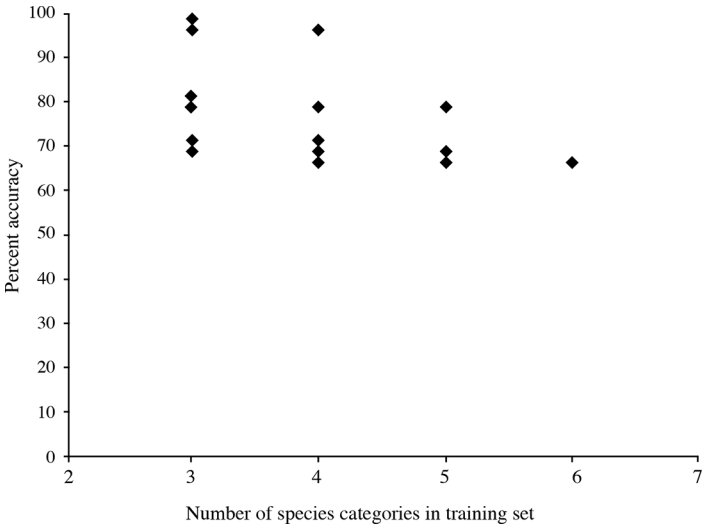

Fig. 4. Percent classification accuracy of ShellBi when classifying images of *C. virginica* shells using training sets with different numbers of species categories (see Table 4 for details). Training sets of 3-, 4-, 5-, and 6-species categories were comprised of hatchery-reared *C. virginica*, and the following species reared in the laboratory: *I. recurvum*, *M. lateralis*, *M. leucophaeata*, *T. plebeius*, and *R. cuneata*. Diamonds represent training sets, each with a different set of species comprising the categories in the training set.

(3) Four training sets composed of *C. virginica*, *M. lateralis*, and *T. plebeius* (250 images for each species) were created. All training sets contained the same (250) images of *M. lateralis* and *T. plebeius*. Images in the *C. virginica* category were varied to form the four different training sets, which were comprised of images of larvae raised: 1) in the hatchery in 2009, 2) in the hatchery in 2009 and 2010, 3) in the hatchery in 2009, 2010, and 2011, and 4) in the hatchery in 2009, 2010, and 2011 and images of *C. virginica* larvae from the warm and cool treatments of the experiment (Tables 3 and 5). The mean temperature and salinity at which the larvae were raised in each training set were 1) 25.4°C ± 1.6 and 10.6 ± 0.4 (n = 30), 2) 26.6°C ± 2.3 and 11.2 ± 0.4 (n = 60), 3) 25.9°C ± 1.1 and 9.1 ± 0.2 (n = 90), and 4) 25.3°C ± 2.3 and 13.2 ± 0.4 (n = 153), respectively. These training sets were used to classify the same unknown set, which was composed of images of *C. virginica* from the warm and cool treatments of the experiment (n = 424). Results indicate that as the variation in growth conditions increased within the *C. virginica* portion of the training set, classification accuracies increased from 76.7% to 98.5% (Table 6). In a second test, a 6-species training set and the 3-category training set based on taxonomic order (Ostreoida, Veneroida, Mytiloida) were used, with some (n = 100) of the *C. virginica* images replaced with those from the warm and cool treatments. These training sets were employed to classify the same unknown set used in the test in the previous experiment, which was composed of other images of *C. virginica* from

Table 5. The number of images of *C. virginica* larvae grown in different temperature and salinity treatments which were added to the *C. virginica* training set category denoted by CV* in Tables 3 and 4. Mean, standard deviation, and sample size for temperature and salinity measurements are reported.

Source	Temperature	Salinity	Number of images
Experimental chamber	27.9 ± 0.7 (*n* = 12)	10.3 ± 0.7 (*n* = 25)	8
Experimental chamber	27.7 ± 0.6 (*n* = 17)	14.1 ± 0.6 (*n* = 32)	27
Experimental chamber	27.5 ± 0.6 (*n* = 15)	14.4 ± 0.7 (*n* = 30)	12
Experimental chamber	27.6 ± 0.6 (*n* = 20)	20.5 ± 1.0 (*n* = 50)	20
Experimental chamber	21.1 ± 1.0 (*n* = 13)	10.3 ± 0.7 (*n* = 25)	19
Experimental chamber	20.9 ± 1.0 (*n* = 15)	14.1 ± 0.6 (*n* = 32)	34
Experimental chamber	21.4 ± 1.0 (*n* = 15)	14.4 ± 0.7 (*n* = 30)	6
Experimental chamber	22.7 ± 1.0 (*n* = 16)	20.5 ± 1.0 (*n* = 50)	19
Hatchery	25.9 ± 1.0 (*n* = 30)	10.3 ± 0.9 (*n* = 30)	105
TOTAL			250

Table 6. Percent classification accuracy using four training sets to identify "unknown" *C. virginica* larvae that were raised in the experiment. The training sets were composed images of *M. lateralis*, *T. plebeius*, and *C. virginica*, the latter of which were varied to incorporate larvae grown in different conditions: 1) in the hatchery in 2009 (CV-2009), 2) in the hatchery in 2009 and 2010 (CV-2009-2010), 3) in the hatchery in 2009, 2010, and 2011 (CV-2009-2010-2011), and 4) in the hatchery in 2009, 2010, and 2011, and in the temperature-controlled experiment (CV-2009-2010-2011-exp).

Training set	Percent accuracy
CV-2009	76.7
CV-2009-2010	76.8
CV-2009-2010-2011	84.7
CV-2009-2010-2011-exp	98.5

the warm and cool treatment of the experiment (*n* = 424). When larvae from the experiment were added to the *C. virginica* portion of the training set, classification accuracy with the 6-category training set improved from 66.8% to 97.1%. Classification accuracies with the 3-category training set were slightly higher than those with the 6-category training set, improving from 87.8% to 98.3% when images of larvae from the experiment were included in the training set. Based on these findings, it is recommended that the images of larvae used to create training sets be representative of the growth conditions of larvae in need of identification, especially in terms of temperature and salinity.

Estimating misclassification rates

Classification tests were performed to determine how well the ShellBi method could identify the target species *C. virginica* given various proportions in a sample. Two training sets were used: a 6-species training set composed of 250 images each of *C. virginica*, *M. lateralis*, *T. plebeius*, *R. cuneata*, *M. leucophaeata*, and *I. recurvum* larvae, and a 3-category order-based training set, using the same 6 species categorized by taxonomic order [1: Ostreoida, oysters (*C. virginica*), 2: Veneroida, clams (*M. lateralis*, *M. leucophaeata*, *R. cuneata*, *T. plebeius*), 3: Mytiloida, mussels (*I. recurvum*)]. Both training sets contained images of larvae from warm and cool treatments of the experiment to ensure wide variation in growth conditions within the training sets (Tables 3 and 5). Three different groups of unknown sets were classified: 1) *C. virginica*, *T. plebeius*, and *M. lateralis*, 2) *C. virginica*, *T. plebeius*, and *I. recurvum*, and 3) *C. virginica*, *R. cuneata*, and *M. lateralis*. Each group contained 7 sets of 100 images of "unknown" larvae in which the percentage of images of *C. virginica* varied (2%, 10%, 25%, 33%, 50%, 75%, and 90%), with the remaining percentages comprised of equal number of images of two other species. Indices of classifier performance were calculated based on the actual number of *C. virginica* images and on true positives, false positives, and false negatives for *C. virginica*. A true positive occurs when an image of *C. virginica* is classified as *C. virginica*. A false positive occurs when an image of a species other than *C. virginica* is classified as *C. virginica*. A false negative occurs when an image of *C. virginica* is misclassified as any other species. Probability of detection (i.e., the probability that the classifier will identify images correctly, P_D = true positive counts/[true positive counts + false negative counts] [Hu and Davis 2006]), specificity (i.e., the probability that the classifier's prediction is correct for each category, SP = true positive counts/[true positive counts + false positive counts] [Baldi and Brunak 2001]), and the ratios of false positives and false negatives to the actual number of *C. virginica* images (e.g., if a sample had 2 images of *C. virginica* and 4 images of mussels were classified as *C. virginica*, then the false positive ratio would be 4:2 or 2.0) were calculated. All indices of classifier performance (P_D, SP, false positive and false negative ratios) were calculated for the 3-category and 6-species training sets, which were applied to each of the unknown groups.

Use of the order-based training set resulted in a similar number of misclassifications as the 6-species training set,

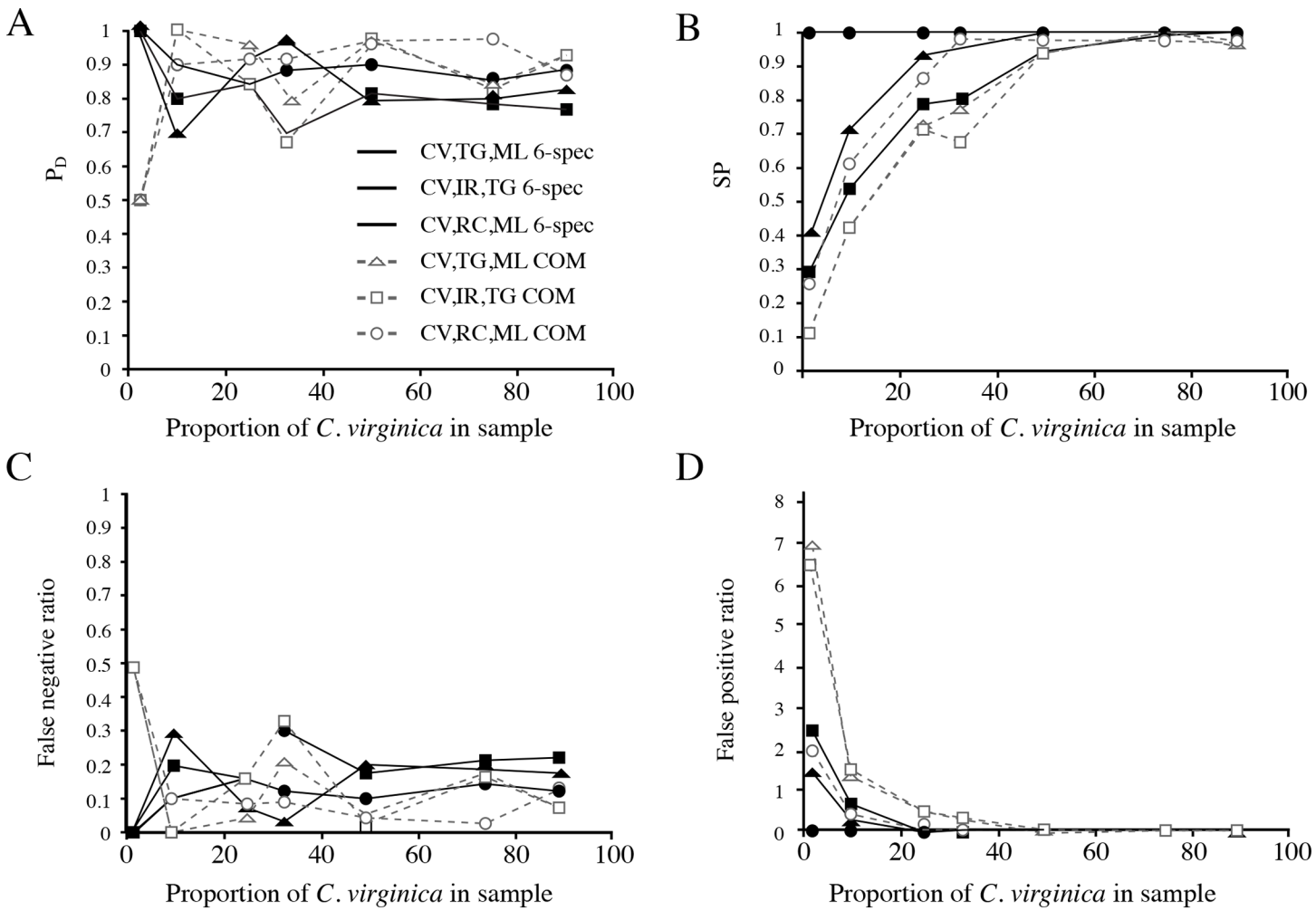

Fig. 5. Misclassification metrics versus the proportion of *C. virginia* (CV) images in a sample: A) probability of detection (P_D), B) specificity (SP), C) the ratio of false negatives to actual *C. virginica* images, and D) the ratio of false positives to actual *C. virginica* images. For all panels, two training sets were used to classify 3 groups of unknown larvae in different proportions. A 6-species training set (6-spec, solid lines) was composed of six categories, each for a separate species: *C. virginica*, *I. recurvum*, *M. lateralis*, *M. leucophaeata*, *R. cuneata*, and *T. plebeius*. A second training set (order-based, dotted lines) contained images of these species grouped by order (clams: *M. lateralis*, *M. leucophaeata*, *R. cuneata*, *T. plebeius*; oyster: *C. virginica*, mussel: *I. recurvum*). These training sets were used to classify three different groups of images of "unknown" larvae: 1) *C. virginica*, *T. plebeius*, and *M. lateralis* (CV, TG, ML), 2) *C. virginica*, *T. plebieus*, and *I. recurvum* (CV, TG, IR), and 3) *C. virginica*, *R. cuneata*, and *M. lateralis* (CV, RC, ML). Each group contained "unknown" sets of images in which the percentage of *C. virginica* in the set ranged from 2% to 90%.

except when the proportion of images of *C. virginica* in a sample was very low (Fig.!5). The probability of detection (P_D) was generally equal or higher for classifications by the order-based training set than for the 6-species training set except when the proportion of images of *C. virginica* comprised 2% of the sample (Fig. 5A). Specificity increased for both training sets as the proportion of images of *C. virginica* in a sample increased, with the 6-species training set performing slightly better when the number of *C. virginica* was high (Fig. 5B). False negative ratios did not exceed 0.33 except for the order-based training set when it was used to classify low percentages of *C. virginica* (2%) (Fig. 5C). The ratio of false positives to actual numbers was higher with the order-based training set when there were relatively few images of *C. virginica* in a sample (Fig. 5D), but this corresponded to a low number of misclassified images (3-8). These metrics show that higher proportions of *C. virginica* in a sample will result in greater classification accuracy, particularly with the order-based training set.

The highest number of false positive and false negative misclassifications from each training set was used to construct confidence intervals that depict the misclassifications that can be expected for different proportions of *C. virginica* in a sample (Fig.!6). The actual *C. virginica* images present plus the highest number of false positives was used to construct the upper line of the interval and the actual *C. virginica* minus the highest number of false negatives was used to construct the lower line of the interval. The confidence interval for the 6-species training set varied from < 5% error at low percentages

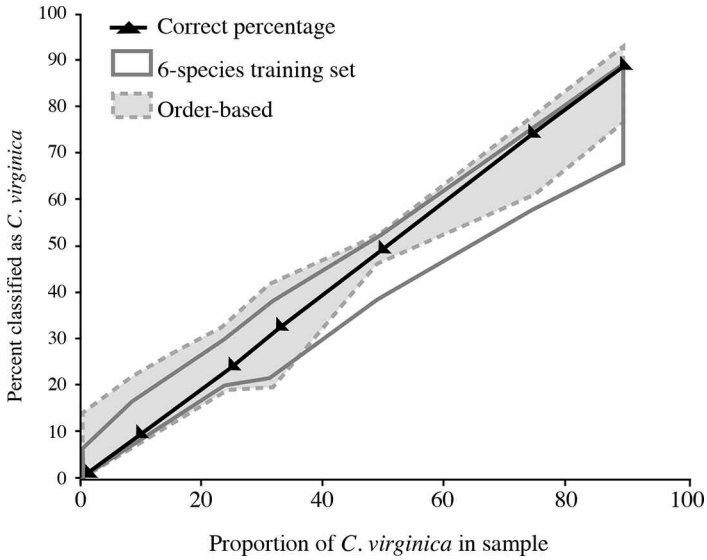

Fig. 6. Classification confidence intervals for the 6-species (no fill with solid gray line) and order-based (gray shading with dashed gray line) training sets. Confidence intervals were constructed around the correct percentage of *C. virginica* classified in a sample (solid line with triangles) using the highest number of false positives and false negatives from tests summarized in Fig. 5. False positives were added to the correct number of *C. virginica* images to construct the top lines and false negatives were subtracted from the correct number of *C. virginica* images to construct the bottom lines. The closer the gray lines are to the black line, the smaller the classification error, which ranged from 5% to 21% for the 6-species training set and from 1% to 22% for the 3-category order-based training set.

(2% *C. virginica* larvae) to < 21% error at higher percentages (90% *C. virginica* larvae). The higher misclassifications at higher percentages are a result of more *C. virginica* being classified as other bivalves (i.e., false negatives) (Fig. 6). The confidence interval for the 3-category order-based training set varied from < 1% error at low percentages (2% *C. virginica* larvae) to < 22% error at medium percentages (33% *C. virginica* larvae) to < 11% at the highest percentages (90% *C. virginica* larvae). The highest error for the 3-category order-based training set is a combined effect of increased false positives and false negatives in the middle ranges (33% *C. virginica*). Based on these results, it is expected that misclassification rates will be within 5% to 21% for the 6-species training set and within 1% to 22% for the 3-category order-based training set depending on the proportion of *C. virginica* in a given sample.

Discussion

Our evaluation shows that the ShellBi technique can be applied with success to distinguish *C. virginica* larvae from the larvae of other bivalve species that are found in the Choptank River, indicating that this approach has application to different species and systems than the one in which it was developed (Waquoit Bay). Results indicate that 1) classification accuracies can increase by as much as 30% when training sets include images of larvae grown in conditions similar to those that are being classified, 2) accuracies can increase by 69% when larvae of different stages (both D-stage and veligers) are included in training sets, and 3) average accuracies are 15% higher when the number of categories within a training set is three compared with six. Although the first two points are novel and specific to this method, the third point has been shown in other image-processing methods that are used to identify plankton (Davis et al. 2004; Grosjean et al. 2004). Finally, misclassification rates were estimated for our target species *C. virginica*, which suggest that this technique can be applied with error rates from 1% to 22% when proportions of the target organisms in the sample range from 2% to 90% (Fig. 6). Results indicate that further methods development aimed at reducing false positive and negative classification rates is a priority.

Differences in growth conditions based on salinity and temperature influenced median shell heights as well as the accuracy of classifying *C. virginica*. Higher temperatures and salinities correspond to faster growth in *C. virginica* (Kennedy 1996) and influence growth in other bivalve larvae (Chanley 1970; Sundberg and Kennedy 1992). Shell heights of *C. virginica* in warm treatments were larger than those in cool treatments, but were shorter than those of hatchery-reared larvae grown at similar warm temperatures. This could be due to the lower assortment of algae fed to the experimental treatments compared with the diet of hatchery *C. virginica* (Langdon and Newell 1996). Regardless of the cause of variation, our results indicate that using images in training sets of larvae that were grown in similar conditions as the unknown sets resulted in higher classification accuracies. This suggests that differences in growth conditions may influence the formation of the shells of bivalve larvae, and hence alter birefringence patterns and classification accuracies. However, potential changes in shell structure and birefringence patterns under different growth conditions warrants further investigation.

The number of categories in a training set and the composition of species in a training set altered the classification accuracy of *C. virginica*. As the number of training set categories increased from 3 to 6, the average accuracy dropped by ~15%, which is consistent with previous studies (Davis et al. 2004; Grosjean et al. 2004; Thompson et al. 2012a). A training set in which 6 species were grouped into 3 categories based on taxonomic order increased classification accuracy of *C. virginica* from 66.8% to 87.8%. These findings suggest that ShellBi would perform well in systems with low numbers of bivalve species in the plankton at any given time (e.g., a system in which 3 species spawn during spring) or in systems where non-target species can be aggregated into a few (≤3) categories.

The composition of the training set was also important. When used to identify the same unknown set, a training set composed of *C. virginica*, *R. cuneata*, and *T. plebeius* had 69.5% accuracy, whereas one of *C. virginica*, *M. leucophaeata*, and *I. recurvum* had 99.8% accuracy (Table 4). This may be explained, to some degree, because smaller *C. virginica* appear to have

similar colors as later stage *T. plebeius* (Fig. 1). This suggests that some species of bivalves at different stages may have birefringence patterns that are similar, resulting in lower classification accuracies, whereas others have patterns that are more distinct, resulting in higher classification accuracies. Although further investigation is needed to determine how shell patterns compare between species throughout development and influence classification accuracies, grouping similar species into a small number of categories can help improve classification accuracies and could be optimized through a machine-learning technique (Fernandes et al. 2009).

The confidence range for misclassifications that can be expected for different proportions of *C. virginica* in a sample may be a conservative estimate. The training sets used in this study were balanced (contain equal numbers of images in each species category) and the SVM classifier assumes that the unknown set contains equal representations of each category (Provost 2000; Lin et al. 2002), but the proportion of *C. virginica* in our unknown sets was varied. Adjusting the cost function (C parameter) of an SVM can help avoid false positives (Sun et al. 2007) and could result in narrower confidence intervals. Future directions to improve ShellBi include adjusting the cost function given different percentages of target species (*C. virginica*) in a sample.

Although the initial set up of ShellBi requires time and effort, ShellBi is the fastest way to both identify and measure different species of bivalve larvae to date once training sets are established. Microscope techniques require a significant time investment while many molecular techniques require time and expense to set up primer or antibody designs or to sequence adult DNA (Garland and Zimmer 2002; Hendriks et al. 2005). When compared with multiplex PCR, ShellBi is less expensive and time consuming for bivalve larvae because individual larvae do not have to be isolated (Thompson et al. 2012a). Although quantitative PCR can provide some insight into the quantity of bivalve larvae, it does not provide information on the sizes of those larvae, which ShellBi does. Another promising technique is fluorescence *in situ* hybridization with DNA probes (Henzler et al. 2010), but the costs are currently prohibitive for large sampling efforts.

Results of this study suggest that ShellBi has broad applicability for the study of size-specific changes in the distribution and abundance of bivalve larvae in estuarine and marine systems. ShellBi has been used successfully to identify larvae in Waquoit Bay (Thompson et al. 2012b) and is being used to help enhance current understanding of *C. virginica* larval dispersal and connectivity in the Choptank River (Goodwin unpubl. data). This technique could be applied to other ecologically and economically important bivalves, both in the laboratory with samples collected from sediment-laden estuaries or in flow-through systems for underway identification of early stage bivalves in marine waters (the tissues of early-stage larvae do not impede resolving birefringent patterns allowing flow-through imaging under field conditions) (S. Gallager pers. comm.). Furthermore, ShellBi may provide insight into the dynamics of other calcareous organisms with shells that show birefringent patterns under polarized light (e.g., pteropods, Goodwin unpubl. data). Finally, because this image-based approach has the potential to be fully automated, it has promise to radically expand our knowledge of the dynamics of bivalve larvae via in situ monitoring platforms and gliders.

Comments and recommendations

Based on the experiments carried out in this study, several improvements are recommended for future applications and research. The first is to establish training sets with several ages of bivalve larvae reared in a range of environmental conditions similar to the system of study. In addition, we recommend the use of the fewest number of categories in a training set as possible. We found that a 3-category training set based on taxonomic order was slightly more accurate at classifying oyster larvae than a 6-category training set in which each category represented a separate species. It is possible that the species grouped by order (e.g., clam larvae) could be distinguished with a second classification test using categories that correspond to species (e.g., *R. cuneata*, *T. plebeius*, *M. lateralis*, *M. leucophaeata*).

Another recommendation is to ensure that the microscope and camera image capture settings are configured so that the background color in all images is uniform for both training and unknown sets. Thompson et al. (2012a) found that training sets created with different microscope settings were not compatible. We found that major background color differences could negatively affect classification accuracies (results not shown), but that minor background color differences (see Fig. 1) for tests conducted in this manuscript did not result in in poor classification accuracies. To avoid major background color variations, we recommend against using metal brackets for polarizers or full wave compensation plates when they are near the light source of the microscope. Changes in temperature due to heating by the light source can lead to large differences in the background color of images when using metal housings. A nonmetal or plastic housing for a polarizer or wave compensation plate near the light source offers more stable conditions that provide similar background colors between images.

The next step for improving the ShellBi method is to increase the speed of image acquisition, ROI extraction, and classification. For the tests presented here, the microscope stage was moved manually or with a joystick attached to an automated stage before an image was taken. A person can image about 100 larval shells per hour with this approach. Currently, efforts toward automation have been made using an automated camera and stage system that will automatically image an entire slide in 46 min (regardless of the number of shells per slide). With this system, 50% of the larvae in a field sample are being imaged in 46 min (half of two slides), which

is faster and more likely to detect rare species than manual identification, which most often relies on subsamples much smaller than half of the sample. In addition, efforts are underway to automate post processing of the bivalve images with automatic ROI detection, ROI cropping, and classification steps, with care taken to assess and minimize errors that can be introduced by subsampling and automation of image analysis (Bachiller et al. 2012). As these enhancements improve how we apply the ShellBi method, so will our ability to rapidly process samples and to conduct field studies with greater spatial and temporal resolution, thereby increasing our understanding of the occurrence and patterns in the presence of bivalve larvae in the field.

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Appendix 4: Ganju and Defne 2014

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