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Improving a semi-automated classification technique for bivalve larvae: Automated image acquisition and measures of quality control

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Abstract

Bivalve larvae are small (50–400 μm) and difficult to identify using standard microscopy, thus limiting inferences from samples collected in the field. With the advent of ShellBi, an image analysis technique, accurate identification of bivalve larvae is now possible but rapid image acquisition and processing remains a challenge. The objectives of this research were to (1) develop a benchtop automated image acquisition system for use with ShellBi, (2) evaluate the system, and (3) create a protocol that would maintain high classification accuracies for larvae of the eastern oyster, *Crassostrea virginica*. The automated system decreased image acquisition time from 2–13 h to 46 min per slide and resulted in the highest classification accuracies at the lowest tested magnification (7X) and shortest image acquisition time (46 min). Quality control tests indicated that classification accuracies were sensitive to camera and light source settings and that measuring changes in light source and color channel intensities over time was an important part of quality control during routine operations. Validation experiments indicated that under proper settings, automated image acquisition coupled with ShellBi could rapidly classify *C. virginica* larvae with high accuracies (80–93%). Results suggest that this automated image acquisition system coupled with ShellBi can be used to rapidly image plankton samples and classify *C. virginica* larvae allowing for expanded capability to understand bivalve larval ecology in the field. Additionally, the automated system has application for rapidly imaging other planktonic organisms at high magnification.

Ecologically and commercially important shellfish can provide important coastal habitat (Seitz et al. 2014), influence nutrient cycles (Newell 2004; Kellogg et al. 2013), and support local fishing communities (Mann 2001; Hall-Arber et al. 2001). These shellfish, including the eastern oyster *Crassostrea virginica*, often have a transient planktonic larval stage and sessile juvenile and adult phases, (Kennedy 1996; Eversole 2001; Beninger and Le Pennec 2006). However, little is known about the planktonic stage of larvae although it influences the recruitment patterns of a population (Gaines and Roughgarden 1985; Kennedy 1996). Discerning patterns in abundance and changes in distributions of planktonic bivalve larvae requires a large number of samples over space and time (Steele 1989; Wiens 1989). Recently, semi-automated plankton imaging techniques have been developed to expand the spatial and temporal scales of sampling (Grosjean et al. 2004; Benfield et al. 2007; MacLeod et al. 2010; Bachiller et al. 2012; Thompson et al. 2012), with both in situ (e.g., Video Plankton Recorder (Davis et al. 1996), ISIIS (Cowen and Guigand 2008)) and benchtop (e.g., Zooscan (Gorsky et al. 2010) approaches. However, these techniques do not identify bivalve larvae to the species level. One semi-automated imaging technique called ShellBi uses machine learning to identify images of bivalve larvae taken under polarized light (Tiwari and Gallager 2003; Thompson et al. 2012; Goodwin et al. 2014). Manual acquisition of images for use with ShellBi can take up to 12 h per sample (Thompson et al. 2012; Goodwin et al. 2014). More rapid benchtop approaches for image acquisition of plankton are needed to decrease processing time for samples collected from turbid waters like estuaries where it is difficult to capture images in situ due to the attenuation and scatter of light (Guo et al. 2015). The objective of this research was to develop and test such an approach.

Traditional methods of identifying bivalve larvae focus on hinge structures or other morphological cues (Chanley and Andrews 1971; Lutz et al. 1982). These methods often require experts, intensive labor, and are subject to a degree of individual subjectivity (Garland and Zimmer 2002). More rapid molecular techniques have been developed (Hare et al. 2000; Garland and Zimmer 2002; Larsen et al. 2005) but those techniques can be susceptible to contamination and

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misidentification (Larsen et al. 2005; Thompson et al. 2012). More pros and cons of various bivalve identification methods are reviewed in Garland and Zimmer (2002) and Hendriks et al. (2005).

A more recently developed method for identifying bivalve larvae is ShellBi. ShellBi utilizes the color and texture-based features extracted from digital images of bivalve larvae taken under polarized light (Gallager and Tiwari 2008). This method uses an image library, or training set, to classify "unknown" images (for more detail see Thompson et al. 2012). The images of the bivalve larvae are classified using pattern recognition software (Gallager and Tiwari 2008). ShellBi was validated by applying DNA and visual classification methods to bivalve species in Cape Cod, yielding high (98%) classification accuracies for hatchery reared larvae but lower accuracies (63–88%) for field samples (Thompson et al. 2012). Goodwin et al. (2014) showed that classification accuracies could be increased (by up to 20%) when training set images included larvae reared under similar environmental conditions to those being classified. Goodwin et al. (2014) also demonstrated that ShellBi was effective for distinguishing different species of bivalves than those that Thompson et al. (2012) tested, suggesting that this method has broad applicability in estuarine and marine systems.

Although ShellBi offers a quantitative way to identify and measure bivalve larvae, image acquisition speed has been limited to $\sim$100 images h^{-1} (Goodwin et al. 2014) while capturing images of larvae under a microscope by manually moving the stage or by using a joy-stick-assisted motorized stage. Both techniques necessitate substantial time investment of a trained technician especially if target organisms are rare and subsampling is not possible. Automating image acquisition would greatly enhance sample processing speed and enable greater spatial and temporal coverage during field surveys for bivalve larvae. In addition, increased speed of acquisition of high resolution images at high magnification has applications for enhancing surveys of other types of plankton such as copepods and fish eggs.

Another challenge with automated image acquisition is identifying and cropping (selecting) regions of interest (ROI). For ShellBI, the ROI is the shell of a bivalve larva. Currently, cropping for ShellBi is done manually by clicking with a mouse around the ROI. This is necessary because automated ROI detection software is not able to differentiate bivalve larvae from the other birefringent materials like suspended sediment in samples from turbid estuaries.

The main objective of this research was to create an automated image acquisition system which would enable faster image acquisition and improved cropping while maintaining a standard of quality control which enabled consistent and high-accuracy classification of *C. virginica* larvae. Custom software was created that enabled a digital camera and automated stage to image the contents of a Sedgwick-Rafter slide automatically and ROI detection software was improved. The system was tested to determine how magnification, software settings, and other factors affected the classification accuracy of bivalve larvae. Quality control measures were developed to ensure that the image acquisition system captured images with consistent alignment, brightness, and color. Methods for sample preparation and storage were also developed (and described in the "Assessment" section) to enhance sample processing time and ensure preservation of larval shells.

Materials and procedures

This section describes the automated image acquisition system which was combined with ShellBi software to create a rapid system for identifying bivalve larvae. It also includes procedures which were developed to maintain image quality and classification accuracies and to prepare field samples for use with the automated imaging system.

Hardware

An automated image acquisition system was developed that integrated hardware and software components to improve image capture, image processing, and overall sample processing speeds for imaging bivalve larvae. The hardware consisted of an automated stage, digital camera, microscope, and a desktop computer (Fig. 1; Table 1). The automated stage, a Semprex KPL53 Servo motor-controlled stage, was configured with a micro-plate holder that fit Sedgewick-Rafter slides and an aluminum baseplate that was clamped to the benchtop to reduce vibration. An Omax M837PL trinocular inverted polarizing microscope with factory stage removed was bolted to the aluminum baseplate. The microscope was fitted with a polarizer (slides into place over the light source), a condenser (which rotates), and a full wave compensation (λ) plate (slides into place). The microscope was fitted with an ocular of 5X and objective lenses of 4, 10, and 20X. The factory stage was removed and therefore the exact magnification could not be calculated by multiplying the ocular by the objective. Hence, magnification was calculated by imaging an American Optical 2-mm reticle and measuring a 100 μm increment on it. The image of the100 μm increment was converted to pixels using ImageJ software and then the camera conversion factor of 3.45 μm/pixel (specific to the camera used) was applied to calculate actual magnifications of 7, 21, and 41X for objective lenses 4, 10, and 20X, respectively.

An Infinity model 2-3C eight-megapixel digital microscope camera was fitted onto the microscope using a digital camera extension piece (Fig. 1A). The camera was further secured by two metal braces that screwed into the side of the camera and rested tightly at the head of the microscope (Fig. 1B). The braces were secured in place to help maintain camera alignment. Other metal braces were installed at each side of the base of the microscope so that the microscope could be secured to the aluminum baseplate of the automated stage (Fig. 1C). The digital camera and automated stage motor controller were connected to a windows PC desktop computer.

The Semprex KPL53 Servo motor-controlled stage was equipped with x, y, and z directions. The z direction is the vertical height the stage can move toward or away from the objective lenses. The height of the stage was adjusted manually so that 9-d old *C. virginica* larvae were in focus, which resulted in younger (2–4 d) and older (> 14 d old) larvae not being in sharp focus. Some Sedgewick Rafter slides did not provide as level a surface as others when placed in the well plate holder of the automated stage. In order to select the best Sedgwick rafter slides, we measured the vertical height at which D-stage larvae were in focus at the four corners and center of several Sedgewick Rafter slides, and chose to use the slides with the least change in height across the slide for processing samples (< 0.1 mm difference). We did not use the automatic focus in the z direction (which is available with the automated stage) because setting the autofocus could result in additional processing time (up to 30 s) per bivalve and sometimes there were > 1000 bivalves in samples from the field.

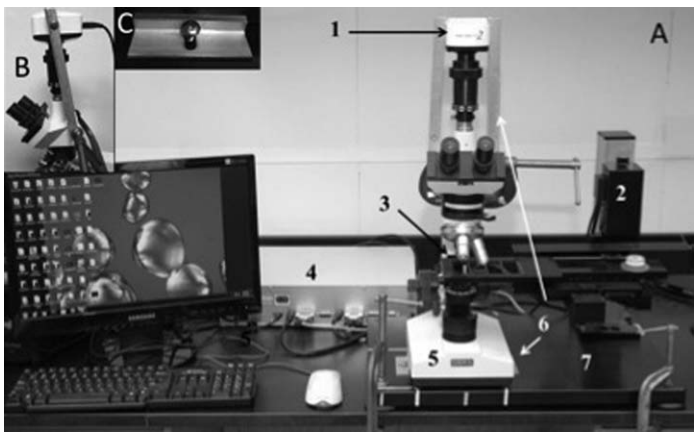

Fig. 1. (A) Automated imaging acquisition system composed of (1) Infinity 2-3C digital microscope camera with metal braces on each side, (2) Semprex automated stage motor, (3) Semprex automated stage with Sedgwick Rafter slide in well plate holder, (4) stage motor controller hub, (5) Omax inverted polarizing microscope with metal braces on each side of the base, (6) four metal braces (two on each side of the microscope), and (7) aluminum baseplate clamped to benchtop. Insets show (B) a side view of one of the aluminum L-channel braces (25.1 × 1.8 cm) that keep the camera and microscope aligned and (C) a close up of one of the aluminum L-channel braces (7.9 × 2.5 cm) that keep the microscope aligned with the baseplate of the automated stage.

Software

Custom software was developed to enable the computer to control both the camera and the automated stage so that images could be captured rapidly. The custom software called on libraries from both the automated stage software (Semprex) and the camera software (Software Development Kit (SDK) from Lumen*era*). The custom software was written in Microsoft Visual Basic .NET (VB.NET). The custom software (available upon request to the corresponding author) made calls to both the stage controller and camera. First the custom software signaled the controller to move the stage to a "home" position. After the home position was reached, the stage was then signaled to move in a series of steps down the length of the Sedgwick Rafter slide and the camera was programmed to capture images at designated points. Between each step the program executed a pause (referred to as settling time) to wait for any vibrations to dampen and then the program called the camera to capture an image. This process was repeated until an entire length of the Sedgwick Rafter cell was imaged. The stage then was programmed to move over the length of one image and capture images in a similar stepwise fashion moving in the opposite direction. This serpentine pattern was repeated until the entire area of the slide was imaged. A binning factor was implemented to speed up image acquisition so that a 4 × 4 pixel square on the camera sensor was summed to become 1 pixel in the final image. This resulted in smaller, brighter images allowing for shorter exposures and less time between successive image captures. Images were saved from the camera in a raw file format to reduce acquisition times by shifting conversion of raw files from the camera to the computer. After imaging the Sedgewick-Rafter slide was complete, the raw image files on the computer were converted into BMP images using software written in visual basic (custom software available upon

Table 1. The components, company, model, and price (United States dollar) of the automated image acquisition system in 2012. The automated stage and Semprex software is available with several options and the price here includes all components needed to run the stage in the *x*, *y*, and *z* planes. The ShellBi software is available and sold with hardware at coastaloceanvision.com.

Component	Company	Model	Price
Digital camera with software	Lumenera/Infinity	2-3C	$2,300
Automated stage with software	Semprex	AMICron 3.2 software w/KPL53 stage	$11,663.9
ShellBi software			
Trinocular polarizing microscope	microscope.net	M837PL	$1,299.99
SDK Lumenera software	Lumenara/Infinity	SDK 2011	$695
Desktop computer	Dell	Optiplex 7010	$917.73
Sedgwick Rafter gridded/ungridded	Cole-Parmer	1801-A10/G20	$135/48 each
Total			$17,059.62

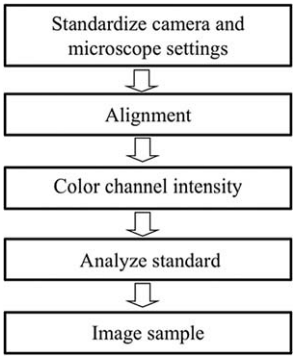

Fig. 2. Flow diagram of system quality assurance and processing steps: (1) standardize the camera and microscope settings for the project (check weekly), (2) align the camera so no areas of the slide are missed (check weekly), (3) measure the color channel intensity (daily), (4) analyze a standard (weekly), and (5) image samples after steps 1-4 are completed.

request to the corresponding author). This post-acquisition image processing was implemented to reduce the time needed to capture images and thereby speed up the image acquisition process. After post-processing, the images were ready to be cropped, measured, and classified with the ShellBi software package. The ShellBi software is available with Continuous Particle Imaging and Classification system (CPICS) at coastaloceanvision.com.

Procedures

Recommended steps and procedures were created to optimize the automated image acquisition system and ensure quality control. These steps were (1) standardize microscope and camera settings, (2) maintain alignment, (3) create training set, (4) check color channel intensity, and (5) classify a standard. These steps may be modified or generalized for applications of this technology to other species of interest (Fig. 2). For example, the classification of a standard may be conducted using other software programs for different species. In addition to these procedures, recommendations for how to prepare and store field samples for image acquisition were developed.

Standardize microscope and camera settings

Optimal microscope and camera settings were determined and then remained fixed so that consistent images were taken for training sets and unknown specimens. For the microscope, the objective lens, the light source, and the rotation of the condenser were set. The rotation of the condenser was set by sliding the polarizer into place, focusing on a bivalve shell under full light extinction, and then rotating the condenser until a black cross formed on the shell (see Tiwari and Gallager 2003). Once full extinction was reached, a lambda (λ) plate was then inserted and a magenta background became apparent. The 4X objective lens was used (see "Assessment" section for explanation). The light intensity level was controlled using a dial near the base of the microscope. A white line was marked on the base of the scope just above the dial position to ensure the dial did not move from this position. The camera settings were originally chosen in the Infinity Analyze software program which allowed each setting to be named and saved within the program. The settings were then programmed into the custom software (available with the custom software from the corresponding author) where they were saved.

Maintain alignment

In order to ensure that a Sedgewick Rafter slide was entirely imaged (and therefor all organisms on the slide would be imaged), an alignment protocol was established after the initial alignment of the system was complete (see Semprex manual for initial set up). A Sedgewick Rafter slide with grid lines was used. After an entire gridded Sedgwick Rafter slide was imaged ($n = 1920$ images), the images were stitched together in a mosaic MATLAB (R2012b) software available at: http://northweb.hpl.umces.edu/open_source_code/open_source_code.htm. The mosaic was examined by zooming in on the grid line sections of each slide and checked to (1) ensure that the entire area of the Sedgewick Rafter slide was captured and (2) the grid lines on the slide lined up across the image. If the entire slide was not imaged, the "home" position was reprogrammed. If the grid lines did not line up, the camera was rotated slightly until proper grid alignment was achieved. This alignment procedure was repeated until the system was aligned (usually no more than 5 times). Based on 52 observations over 100 d, we found that the alignment protocol should be conducted after imaging every 40 samples (a weekly period in our laboratory).

Create training sets

A training set is a group of known images used to classify other images (e.g., Thompson et al. 2012; Demir et al. 2013; Goodwin et al. 2014). Once optimal settings were chosen and saved, training sets of images of specimens of different species of bivalve larvae were created so that they could be used for classifying unknown specimens and for use in quality control of the automated image acquisition system. The training sets were created from laboratory reared specimen (see "Assessment" section for information on the specimen library used to create training sets). At least 200 images were used in each category of all training sets based on Thompson et al. (2012).

Check color channel intensity

ShellBi depends on consistent software and hardware settings to maintain stable accuracies for bivalve larvae identification (Thompson et al. 2012) although some minor fluctuation is tolerable (see Goodwin et al. 2014). Changes in light intensity, specifically color channel intensity, can alter the color of light detected on larval shells as well as the background color of the images. We found that color channel intensity fluctuated over the course of a day and over the lifespan of the light bulb in the Omax microscope.

Therefore a protocol was developed to measure light intensity of individual RGB color channels (red, green, blue) from a bitmap image using code developed in MATLAB (version R2012b). First an acceptable range in variation of color channel intensity was determined, and then a protocol was established to maintain color channel intensities within that range. Color channel intensities were reported as binned values out of a range of 0–255 with 0 being no light and 255 being maximum possible detection. This range was unit-less and was determined by the 8-bit bit depth of each color channel value that made up the output file (in this case a bitmap image).

An "acceptable" light intensity range was determined by measuring the daily variation in color channel intensities and then testing whether the maximum and minimum of these values affected ShellBi classification accuracies for our target organism, *C. virginica*. To measure daily variation in color channel intensities, "blanks" were taken by imaging the light from the light source without a Sedgewick Rafter or any other slide in the stage. Five of the blank images were then analyzed in MATLAB to calculate the color channel intensity of red, blue, green, and overall average values available at: http://northweb.hpl.umces.edu/open_source_code/open_source_code.htm. This process was repeated hourly over the course of 6 d to determine the variability in color channel intensity. The range in red, green, blue, and average color channel intensities was 97.0–115.5, 14.0–15.83, 19.9–23.3, and 43.7–51.6, respectively. To test whether the maximum and minimum of these values affected classification accuracies for *C. virginica*, a training set was created by capturing and cropping images of *C. virginica*, *Ischadium recurvum*, and *Rangia cuneata* ($n = 200$ for each species) when color channel intensity was near the mean of the average color channel intensities (49.9). These three species include variation in genera of bivalves (oyster, mussel, clam) and were selected based on specimens available at the time of the tests. This "quality control" training set was used to classify three sets of 50 images of 9-d old C. *virginca* larvae which had been captured at the mean, maximum, and minimum of the daily range in color channel intensities and then cropped. Classification accuracies of *C. virginica* ranged from 92% to 100%, indicating that the maximum and minimum in daily color channel intensity fluctuations did not cause unacceptable decreases in classification accuracies. Therefore, the range in red, green, blue, and average color channel intensities reported above was selected to be the "acceptable" range.

After the acceptable range in color channel intensities was determined, a protocol was established to ensure images were captured with color channel intensities in this range. Five blanks were captured and analyzed three times per day to ensure color channel intensity values remained within the acceptable range. If the color channel intensities were not within the acceptable ranges, the intensity of the light source was adjusted until they were within the acceptable range or the bulb was replaced on the microscope.

Repeated classification of a standard

A performance-based test was conducted to ensure quality control for the automated image acquisition system. Specifically, 50 images of 9-d-old *C. virginica* larvae were imaged once per week, cropped, and then classified using the ShellBi software with the "quality control" training set (described above). This helped ensure that high classification accuracies were maintained over the 3 months that field samples were being imaged.

Preparing field samples for image acquisition

New protocols were developed for preparing field samples for imaging bivalve larvae by reducing the number of other organisms and small sediment particles present in the sample and by removing tissue of the larvae which inhibits detection of birefringent patterns in veliger and pediveliger larvae (see Supporting Information Fig. S.1 for summary instructions). Samples collected from the Choptank River were stored in 200 mL jars with 4% formalin seawater solution buffered with sodium borate. Under a fume hood, a sample was poured through a 350 μm sieve into a 300 mL beaker to remove larger particles. The sample in the beaker was poured through a 44 μm sieve. The 44 μm sieve was rinsed using 40% bleach and 60% Deionized (DI) water buffered with sodium borate into a centrifuge tube. The sample was left for 20 min to digest tissue and break apart valve hinges and then poured through another 44 μm sieve. The sample was then rinsed from the sieve into a 15 mL centrifuge tube using buffered DI water (buffered with sodium borate) and left for 5 min to settle (the time it took for the smallest shells to sink to the bottom). The supernatant was carefully pipetted off until a 2 mL sample volume was left in the tube. The supernatant was discarded after observing that no bivalve larvae were present ($n = 270$). The remaining sample was mixed and resuspended within the 2 mL of solution by pipetting the sample up and down 3–4 times within the centrifuge tube (in an up and down fashion avoiding circular motion). Then a 1-mL aliquot was pipetted from the centrifuge tube and placed (from left to right) onto the center of a Sedgewick Rafter slide (non-gridded). A coverslip was carefully placed on top of the Sedgwick Rafter slide and the slide was then placed in a well plate holder on the automated stage. The remaining 1-mL aliquot was pipetted onto another Sedgewick Rafter slide in the same manner.

Subsamples of the two aliquots were conducted by imaging half of the Sedgwick Rafter slide (lengthwise). Tests performed indicated that the first 1-mL aliquot pipetted onto the Sedgewick Rafter slide had unequal numbers of larvae compared with the second aliquot, but that there was no statistically significant difference in the number of bivalve shells on the left compared to the right half of each slide (Students *t*-test, $p = 0.37$, $n = 60$). Therefore, half of each slide

with the first and second 1-mL aliquots was imaged. By imaging half of each slide in 23 min (including start up time), 50% of the sample was imaged. Note that the count of larvae in a plankton sample would be calculated as two times the number of ROIs (to take into account the 50% subsampling) divided by two (to take into account the fact that each larva had two shells). Images of known specimens that were reared to create training sets underwent the same procedure, except that sieving was not necessary and full slides (rather than 50%) were imaged.

Sample storage

Sample storage considerations are important for this method. Although Goodwin et al. (2014) found no difference in classification accuracy using ShellBi when samples were preserved in buffered 95% ethanol or buffered 4% formaldehyde, buffered 4% formaldehyde solution should be used for long term storage >2 yr because shells of bivalve larvae stored in buffered 95% ethanol cracked after 2 yr (Goodwin, Thompson pers. obs.). To prepare samples for long term storage, the samples should be preserved with 4% formaldehyde buffered to a pH of 8.0–8.1with sodium borate (pH 10.1). The pH of the samples should be monitored over time. O'Meara et al. (2013) found that birefringence is lost on veliger mussel (*Dreissena bugensis*) larvae if they are not stored in basic (pH 7.0–9.0) conditions. However, larval shells can dissolve when sample pH drops below 8.0 (Goodwin, pers. obs). Therefore, pH should be tested 1 or 2 d after sample collection, after the first week, after the first month and then quarterly thereafter. If pH drops near or below 8.0, buffer should be added to bring pH back up to the 8.0–8.1 ranges, then retested a few days to a week later to ensure that the pH remains stable.

Assessment

Tests were conducted to evaluate the automated image acquisition system and improvements to the ShellBi software in order to attain optimum classification accuracies. Specifically, magnification and image resolution, color channel intensity, ROI detection, and camera software settings were assessed. Finally, two blind validation experiments (Validation One and Validation Two) were conducted to test classification accuracies of the automated image acquisition system.

The shells of bivalve larvae that were used in these tests were derived from a collection of known specimens of bivalve larvae that we assembled. Seven bivalve species, which are found in Choptank River at the same time as our target species *C. virginica*, were spawned and reared and images of their shells, and those of *C. virginica*, were captured. The adult bivalves that were collected from the Choptank River and reared in the laboratory consisted of: *I. recurvum* (hooked mussel), *Mulinia lateralis* (dwarf surf clam), *Mytilopsis leucophaeata* (dark false mussel), *Macoma mitchelli* (matagora macoma clam), *R. cuneata* (Atlantic rangia clam), and *Tagelus plebeius* (razor clam). Larvae of *C. virginica* (eastern oyster) were obtained from the Horn Point Hatchery and *Guekensia demissa* (marsh mussel) were obtained from the Rutgers Aquaculture Innovation Center. Spawning and rearing procedures were consistent with summer conditions in Choptank River and were explained in detail (see Goodwin et al. 2014) for all species with the exception *G. demissa*. The *G. demissa* larvae were reared in conditions similar to Delaware Bay at a temperature of 24.9°C at a salinity of 22.5 and fed *Isochrysis galbana*, *Pavlova lutheri*, and *Chaetoceros calcitrans*. Because the number of specimens available for testing differed between species and changed over time, some of the assessment tests were conducted with a subset of the eight species (see Table 2 for details). Also, multiple bivalve species were used as target organisms in some tests, in addition to *C. virginica*, to better assess the capabilities of the automated image acquisition system and software.

Magnification and image resolution tests

The objectives of the magnification and image resolution tests were (1) to choose the lowest magnification that resulted in high classification accuracies and the fastest image acquisition time, and (2) to determine how changes in image resolution within the ShellBi software influenced classification accuracies. Previous research with the ShellBi technique was conducted at a magnification of 50X (Thompson et al. 2012; Goodwin et al. 2014). To test a range of magnifications, the automated stage and software was used to image bivalve larvae on a Sedgewick Rafter slide at three different magnifications: 7, 21, and 41X. It took 46, 120, and 160 min to image a slide at magnifications of 7, 21, and 41X, respectively. Images of bivalve larvae were captured at each magnification using consistent hardware and software components, except that objective lenses (4, 10, and 20X) were changed to create the different magnifications.

Training sets composed of 200 images of four species of bivalve larvae (*C. virginica*, *I. recurvum*, *R. cuneata*, and *M. leucophaeata*) ($n = 800$ total images) were created and used to classify "unknown" images of each species ($n = 25$ for each species). These training sets were created for each magnification and images of the "unknown" specimens were also captured at each magnification. Both "unknown" and "training" sets included images of D-stage and veliger larvae.

In addition to testing the effect of magnification on classification accuracy, the influence of image resolution within the ShellBi software was also determined. In the research performed by Goodwin et al. (2014), the software did not reduce the resolution of images. The resolution of images taken at different magnifications was reduced by 40%, 20%, and 0% and classification tests were performed to determine the influence of image resolution on classification accuracies.

Table 2. Assessment test information. The table includes the target species of each test, whether classification tests were performed, and, if so, what species were included in the unknown and training sets.

Test	Target species	Classifications performed?	Species in unknown set	Species in training set
Magnification and image resolution	CV, IR, RC, DF	Yes	CV, IR, RC, DF	CV, IR, RC, DF
Color channel intensity	CV	Yes	CV	CV, IR, RC
ROI detection	CV, GF, IR, ML, DF, MM, RC, TP	No	N/A	N/A
Camera software setting performance	CV, IR, RC	Yes	Cv, IR, Rc	CV, IR, RC
Validation One	CV	Yes	CV, GF, IR, ML, DF, MM, RC, TP	CV, GF, IR, ML, DF, MM, RC, TP
Validation Two	CV	Yes	CV, GF, IR, ML, MM, RC, TP	CV, GF, IR, ML, MM, RC, TP

CV, *Crassostrea virginica*; IR, *Ischadium recurvum*; GD, *Geukensia demissa*; DF, *Mytilopsis leucophaeata*; ML, *Mulinia lateralis*; MM, *Macoma mitchelli*; RC, *Rangia cuneata*; TP, *Tagelus plebeius*; and N/A, not applicable.

Classification accuracies of images captured at different magnifications and with different image resolutions ranged from 88% to 100%. Classification accuracy for images taken under magnification settings of 7X were highest for all four species (98–100%) regardless of the reduction setting used (Table 3). There was no change in classification accuracy when image resolution was reduced at a magnification of 7X and $< 1\%$ change in classification accuracy when image resolution was reduced at magnifications of 21X or 41X. Based on the results of these classification tests, it was concluded that the lowest magnification setting of 7X yielded highest (98–100%) accuracies and fastest sample imaging time (46 min) and that reducing image resolution in the software, within the tested limits, did not affect classification accuracy.

Table 3. The percent classification accuracy for four "unknown" bivalve species when images in training and "unknown" sets were captured under different microscope magnifications and when image resolution was reduced prior to classification. Each training set was composed of 200 images of shells of *Crassostrea virginica* (CV), *Ischadium recurvum* (IR), *Rangia cuneata* (RC), and *Mytilopsis leucophaeata* (DF)) for a total of 800 images. The training sets were then used to classify 25 images of shells of CV, IR, RC, and DF as "unknowns." For each test, the training set images and "unknown" images were captured under the same magnification and software reduction setting. The different magnifications were applied by changing the objective lenses on the hardware. Image resolution was reduced within the ShellBi software.

Test	Percent reduction in image resolution (software)	Magnification (hardware)	Percent accuracy CV	DF	IR	RC
1	40	7X	98	98	100	100
2	40	21X	97	95	99	100
3	40	41X	94	88	98	96
4	20	7X	98	98	100	100
5	20	21X	96	95	99	100
6	20	41X	94	88	99	96
7	0	7X	98	98	100	100
8	0	21X	97	96	99	100
9	0	41X	94	89	98	95

Color channel intensity

Over a period of 100 d, color channel intensities were measured and monitored and a standard set of 50 images of 9-d old *C. virginica* larvae were classified (Fig. 3) as part of the protocol for maintaining high classification accuracies (described in the "Procedures" section). Classification accuracies were consistent (98–100%) until the color channel intensity for red, green, and blue dropped. The intensity drop was due to a faulty light bulb which led to lower classification accuracy (70%) of the standard unknown set. The light bulb was replaced and color channel intensity was restored to acceptable levels, as indicated by classification tests (98–100%) (Fig. 3). The degradation of a light bulb or change in a power source over time could also potentially cause lower light intensities. Based on these observations, color channel intensity influences the classification accuracy of ShellBi and should therefore be monitored daily to be maintained within an acceptable range.

ROI detection

Tests were conducted to assess the ability of the updated ROI detection software to automate the post processing of images. Samples ($n = 23$) that included oysters (*C. virginica*), mussels (*G. demissa*, *I. recurvum*), and clams (*M. mitchelli*, *M. lateralis*, *M. leucophaeata*, *R. cuneata*, *T. plebeius*) were imaged with the automated image acquisition system. The larvae that were imaged ranged in ages (2–16 d) and lengths (44–330

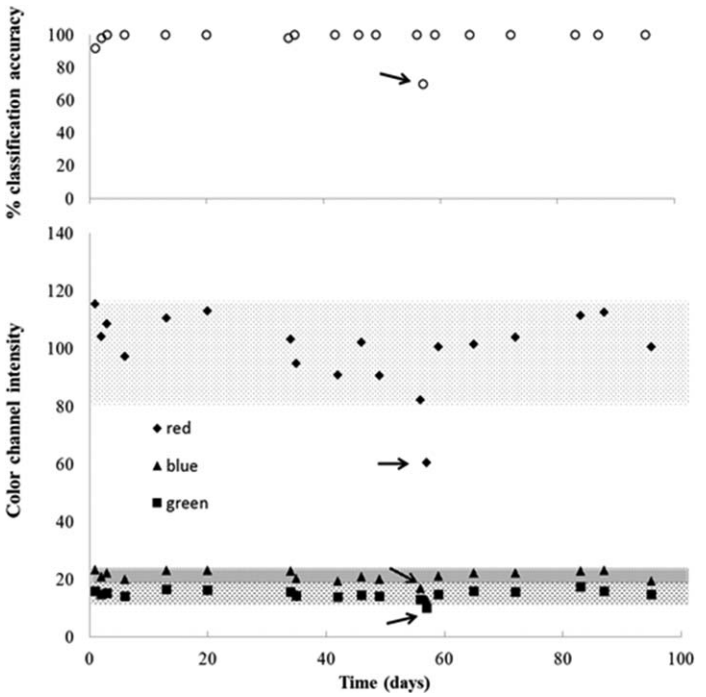

Fig. 3. Percent classification accuracy of 9-d-old *C. virginica* larvae (upper panel) and concurrent color channel intensity measurements (bottom panel) taken over a span of 100 d. Each data point for classification accuracy was the result of classifying 50 images of 9-d-old *C. virginica* using a three species training set (*C. virginica, I. recurvum, and R. cuneata*). The color channel intensity values were calculated using five blanks captured from the automated stage and were compared to the acceptable range (hatched regions) (see "Procedures" section). Arrows indicate the time when color channel intensity values dropped below the acceptable range due to a microscope light bulb malfunction, and when percent classification accuracies also dropped (from an average of 98–70%).

μm). Images with birefringence were sorted into folders using the automated sorting software (MATLAB (R2012b) software available at: http://northweb.hpl.umces.edu/open_source_code/open_source_code.htm). Trained technicians counted the bivalve shells in the folders, after which the automated ROI detection software was used to enumerate the number of ROIs in the images. The same procedure was repeated with 30 samples (200 L^{-1} each) that had been collected from the Choptank River in July of 2012. These samples included clam, mussel, and oyster larvae of various sizes.

The relationship between counts of bivalve larvae generated by the automated ROI detection software and counts by trained technicians was stronger for hatchery-reared specimens ($R^2 = 0.94$, $p = < 0.01$, $n = 33$, JMP version 11.1) than it was for field collected bivalve larvae ($R^2 = 0.56$, $p = 0.11$, $n = 30$, JMP version 11.1) (Fig. 4). For field samples, the automated ROI detection software underestimated the number of bivalve larvae compared to trained technicians because bivalve larvae were not detected as ROIs by the software (Fig. 4). Based on these results, we conclude that this software has use in laboratory and hatchery applications but field samples should be manually cropped until further improvements in the software are made.

Camera software setting performance

Tests were conducted with the automated image acquisition system to determine the influence of camera software settings on the performance of ShellBi. Five different camera settings (labeled 1-5) were created by altering specific attributes in the Infinity Analyze software program (Table 4). Varying the attributes created different background colors in the images (Fig. 5). All settings and attributes were identical except for the exposure, gain, light source setting, saturation, brightness, contrast, and hue. Five three-species training sets composed of 200 images each of *C. virginica*, *I. recurvum*, and *R. cuneata* were created with images taken under the five settings using the automated image acquisition system. A larger training set (labeled "All (1-5)") was constructed as a compilation of the five different training sets ($n = 3000$ images).

The six training sets were used to classify 150 images of "unknown" *C. virginica, I. recurvum*, and *R. cuneata* (50 images of each species) which were taken under each of the five settings, for a total of 30 tests of ShellBi classification accuracy (5 unknown sets $\times$ 6 training sets = 30 tests) (Table 5). Classification accuracies for unknown *C. virginica, I. recurvum, and R. cuneata* ranged from 4% to 100% and differed between species and between camera settings (Table 5). In general, the highest accuracies (82–100%) occurred when the settings of the training sets and those of the "unknown" sets were the same (Table 5, especially overall accuracies reported in Table 5D). The training set composed of images taken under all settings (All (1-5)) had classification accuracies from 85% to 95%. These tests indicate that using the same settings for training sets and the unknown images yielded high overall classification accuracies and that different settings may be optimal for different species.

Validation experiments

Two validation experiments (Validation One and Validation Two) were conducted to assess the accuracy of both the ShellBi software and trained technicians to classify images of shells of bivalve larvae which had been captured using the automated image acquisition system.

Three training sets were constructed with the automated imaging acquisition system and used to classify shells of bivalve larvae with ShellBi in both validation experiments. These training sets were composed of images of eight species of bivalve larvae found in the Choptank River grouped into three categories (oysters: *C. virginica;* mussels: *I. recurvum, G. demissa;* clams: *M. leucophaeata, M. lateralis, M. mitchelli, R. cuneata*, and *T. plebeius*). Each category included images of larvae of different ages. One training set, called COM1000, had 1000 images per category (Supporting Information Table S1) and was taken with camera settings 2 (Table 4). A second

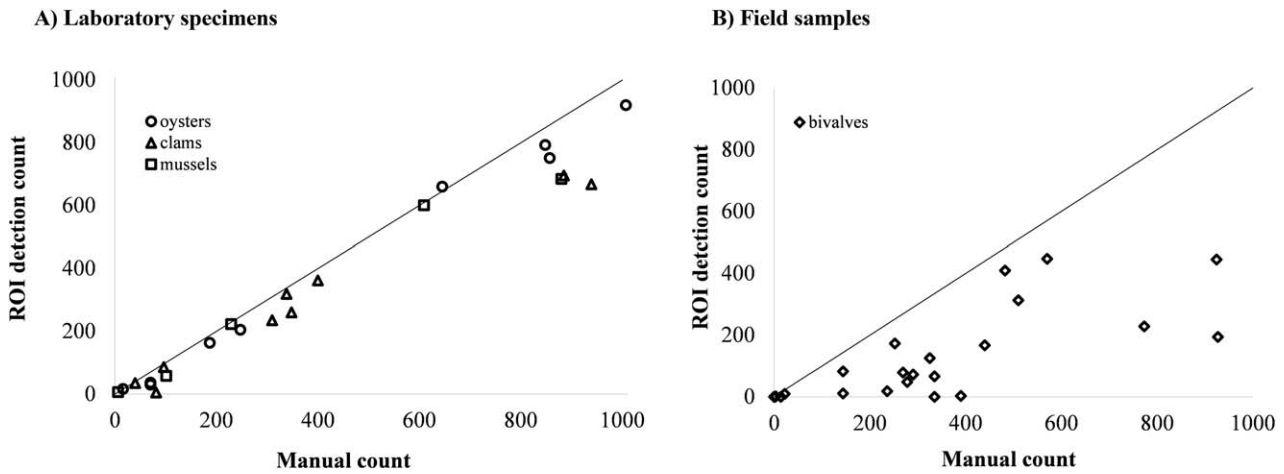

Fig. 4. The number of shells of bivalve larvae in (A) samples containing laboratory specimens ($n = 33$), and (B) field samples ($n = 30$) which were detected by the automated ROI detection software (y-axis) vs. those counted by a trained technician (x-axis). The line indicates a 1 : 1 ratio between counts of bivalve shells by trained technicians and the automated ROI detection software. Both the laboratory specimens and field samples contained species of oyster, clam, and mussel larvae.

Table 4. Software configurations of five different settings for the digital camera. The five settings 1–5 were created by changing attributes in Infinity Analyze software including exposure, gain, gamma, light source, saturation, brightness, contrast and the red and green hues. Note: the actual light source was kept constant but the setting choice for "Light source" in the software program was adjusted. The configuration of blue light (1.0), averaging (1), subsampling (1), interval (1 s), and duration (10 s) were held constant across settings.

Setting name	Exposure	Gain	Gamma	Light source	Saturation	Brightness	Contrast	Red	Green
1	151.0	10.6	0.82	Fluorescent	1.31	4	4	1.0	1.0
2	151.0	15.2	0.82	Fluorescent	1.31	4	4	1.0	1.0
3	89.1	21.4	0.82	Incandescent	1.00	0	0	1.0	1.0
4	84.5	15.2	0.82	Incandescent	1.31	4	4	1.3	1.3
5	270.8	4.4	1.4	Fluorescent	1.00	5	28	1.0	1.0

training set (COM700) was composed of images using setting 1 (Table 4). Furthermore, COM700 contained fewer total images of bivalves (700 images per category), fewer images of *M. lateralis* and *T. plebeius* and no images of *G. demissa* due to limited availability. However, different ages and species were all represented as equally as possible (Supporting Information Table S1). The third training set (COM1700) was simply a combination of COM700 and COM1000, so that each category had 1700 images.

Each validation experiment consisted of 18 separate tests of the ability of trained technicians and ShellBi to identify images of *C. virginica* from those of other bivalves. The 18 tests were each composed of 100 "unknown" images ($n = 1800$ total unknown images). Unknown images for Validation One were taken under the same settings as the training set COM1000. Unknown images for Validation Two were taken under the same settings as COM700. For each experiment, a lab member (who did not undertake classifications) created 18 folders which contained 100 images of different ages of *C. virginica, I. recurvum, G. demissa, M. leucophaeata, M. lateralis, M. mitchelli, R. cuneata*, and *T. plebeius*. Care was taken to vary the number of images of species and ages (sizes) to simulate differences that might be found in field samples (e.g., the number of *C. virginica* in folders ranged from zero to 44). For Validation Two, there were no *G. demissa* images and fewer *M. lateralis* and *T. plebeius* because specimens were not available (Supporting Information Table S1). The original folders that contained the species name and age were stored on a password protected secure server. For the validation experiments, a copy of the 18 folders was created and all images were renamed so that the identity and ages of the bivalves would not be known by the trained technicians who undertook the classifications.

Two trained technicians used the training sets COM1000 (for Validation One) and COM700 (for Validation Two) as visual keys to assist with identifying images of *C. virginica*

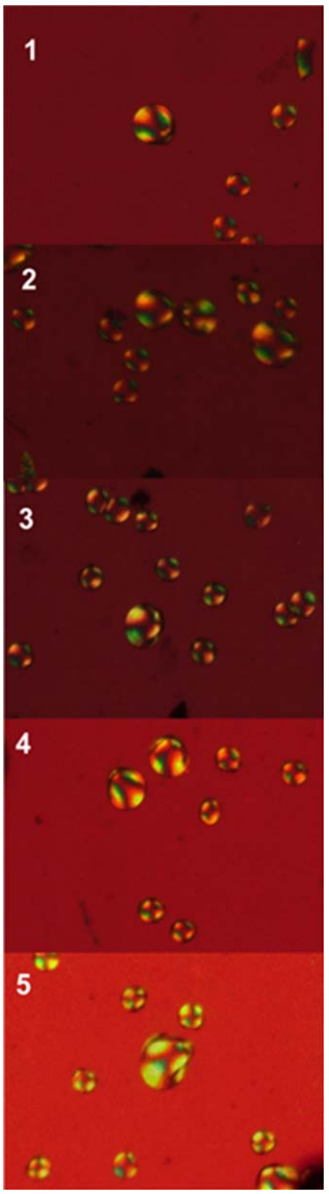

Fig. 5. Images 1–5 contain 4- and 9-d-old larvae of *C. virginica* and correspond to the camera settings 1–5 (details in Table 4) which were used for tests reported in Table 5. Setting differences were created by altering attributes in the camera software Infinity Analyze.

larvae within the 18 folders for each experiment. Misclassification was assessed by comparing the images that the technicians identified as *C. virginica* with those in the original 18 folders and noting if any *C. virginica* images were not correctly identified.

In addition, images in each of the 18 folders were classified using ShellBi and each of the three training sets. Misclassifications for ShellBi were calculated using a script written in MATLAB that calculated true positives (a true positive was an image of *C. virginica* that was properly classified as *C. virginica*).

Trained technicians were able to classify images of *C. virginica* larvae with high accuracies (> 92% on average for both validation experiments) (Table 6). In contrast, classification of images of *C. virginica* with ShellBi ranged from averages of 60–94% for both experiments. ShellBi had highest accuracies (80–93% on average) when training sets contained images of larvae that were taken under the same settings as those of the "unknown" images. Accuracies dropped (60–74% on average) when training sets were used to classify "unknown images" that had been taken under different settings (Table 6). Based on these tests, we conclude that the automated image acquisition system can capture images which can be classified with high accuracy by a trained technician. In addition, this system can be used with ShellBi to successfully classify images with high speed and accuracies > 85% on average for *C. virginica* larvae, as long as camera settings used to create training sets and unknown images correspond. We recommend that trained technicians check and correct ShellBi classifications for all field samples processed, thereby ensuring high accuracies while taking advantage of the rapid image classification by the ShellBi software.

Discussion

Our goal was to create an automated image acquisition system and improve ShellBi software to rapidly and accurately identify and measure larvae of a target bivalve species (*C. virginica*). Results indicate that the automated image acquisition system at 7X magnification was able to image an entire Sedgewick Rafter slide in 46 min. In addition, the ShellBi software distinguished *C. virginica* larvae that were imaged with the system with high accuracies (> 85% on average) which could be improved to > 92% on average if a trained technician were to check and correct the computer-based classifications. The automated image acquisition system enabled an increase in sample processing time that was up to 12 h per sample faster than previous efforts where manual image acquisition was used (Goodwin et al. 2014). This increase in speed may help offset hourly technician costs when processing large numbers of samples.

Our research showed that higher accuracies and faster processing times could be achieved with lower (7X) magnifications. Previous research with the ShellBi technique was conducted at magnifications of 50X (Thompson et al. 2012; Goodwin et al. 2014). The higher accuracy when using lower magnification may stem from the Support Vector Machine that the ShellBi software uses to distinguish between species. It could work better with less information because details in texture features and color angles are smoothed out at lower resolutions. Further testing may reveal that magnifications lower than 7X could be equally accurate, although there may be a point at which lower magnifications would have

Table 5. Classification accuracies for images of shells of (A) *C. virginica*, (B) *I. recurvum*, (C) *R. cuneata*, and (D) all three bivalves combined when classified under five different camera settings (1-5). Training sets (rows) were used to classify "unknown" sets (columns). Each group was imaged under different camera settings (1-5, described in Table 4). The sixth training set, "All1-5," was composed of images captured at all five settings. Shaded regions indicated accuracies $\geq 90\%$.

		Unknown set				
	Training set	**1**	**2**	**3**	**4**	**5**
A) *C. virginca*	1	92	94	84	88	26
	2	84	94	66	66	94
	3	88	82	96	96	42
	4	4	80	52	84	18
	5	4	92	32	16	94
	All(1-5)	96	88	96	88	88
B) *I. recruvum*		1	2	3	4	5
	1	90	82	90	72	88
	2	70	88	82	80	88
	3	84	74	90	74	84
	4	98	54	54	94	60
	5	98	66	90	44	82
	All(1-5)	88	84	86	84	84
C) *R. cuneata*		1	2	3	4	5
	1	100	98	100	2	92
	2	48	98	78	0	12
	3	90	96	96	4	76
	4	0	0	0	100	6
	5	40	94	50	38	100
	All(1-5)	100	84	100	84	84
D) All species		1	2	3	4	5
	1	94	91	91	54	69
	2	67	93	75	49	65
	3	87	84	94	58	67
	4	34	45	35	93	28
	5	47	84	57	33	92
	All(1-5)	95	85	94	85	85

too little information to distinguish larvae, especially for small D-stage larvae.

The automated ROI detection performed well for laboratory-reared samples but poorly for field samples (Fig. 4). This was mostly due to other (non-bivalve) birefringent material (like some sediment particles) in the samples from the eutrophic Choptank River (Fig. 6). This material confounded the edge detection and ROI extraction process. Performance may be better in oligotrophic waters than in estuaries because turbid waters of estuaries have more attenuation and scatter of light (Guo et al. 2015) and higher suspended sediment concentrations. Further improvements in the software may enable more accurate ROI detection and automated cropping. Newer ROI detection methods are constantly being developed for many applications in the medical field (Jen and Yu 2015; Molder et al. 2015; Vishrutha and Ravishankar 2015). Improvements could help ROI extraction for ShellBi and should be evaluated in future studies. However, without any further advances, our automated ROI detection software could be used "as is" in laboratory or hatchery conditions to rapidly count for bivalve larvae.

Bachiller et al. (2012) recommended an internal control mechanism to check the quality of the procedure used for counting and classifying zooplankton (or bivalve larvae in this case) given all of the rapid development of imaged-based methods. We set up an internal control method by establishing a standard set of 9-d-old *C. virginica* larvae to be classified weekly by a previously established training set (Fig. 3). This system proved to be very useful to gauge hardware consistency with a performance based metric (classification). In addition, alignment protocols were established to ensure accurate counts of bivalve larvae.

Camera settings were an important determinant of classification accuracy for this method. Training sets used to classify unknown sets imaged under the same camera setting were more accurate than training sets used to image unknowns under different settings (Table 5). The training sets taken under each of the five settings resulted in high classification accuracies of the unknown groups imaged under the corresponding five settings, despite differences between settings (Fig. 5), which suggests that the ShellBi technique is robust even when camera settings vary. The tests also indicate that different settings may perform better for other target species. For example, settings 1 and 3 had $>90\%$ accuracies for all three bivalve species tested while settings 4 and 5 resulted in very high accuracies for *R. cuneata* (100%) but $<90\%$ accuracy for *I. recurvum* (82% with setting 5) or *C. virginica* (84% with setting 4) (Table 5A–C). Therefore, we recommend that settings should be tested for target species so that optimal classification performance can be achieved.

Thompson et al. (2012) have shown that the ShellBi technique can be more accurate than PCR and much faster than traditional light microscopy techniques (Carriker 1996) although the latter may still be the most accurate way to identify bivalve larvae to date. The initial set up of ShellBi requires the establishment of known training sets composed of larvae that were reared in similar conditions to the larvae being sampled and identified from the field which can be labor intensive (Thompson et al. 2012; Goodwin et al. 2014). Imaging a sample took 46 min using the automated stage regardless of the quantity of bivalves on a slide. Manually counting samples on a slide ranged from 0.5 h (with no bivalves present) up to 13 h with hundreds present (pers. obs.). Therefore, this technique has the potential to reduce imaging time (and technician hours) by up to 12 h per sample. After a specimen library is established, capturing images with our automated image acquisition system and classifying

Table 6. Classification accuracies for images of shells of *C. virginica* from two validation experiments. Each validation experiment contained multiple tests that were designed to compare classification accuracies by trained technicians with the ShellBi classification software. In each test, 100 images of shells of eight species of bivalve larvae, with varying numbers of *C. virginica* shells, were classified. For the tests of the ShellBi software, three different training sets (COM700, COM1000, and COM1700) were used which contained images captured under different microscope settings. The image capture settings for COM1000 matched the settings at which the "unknown" images in Validation One were captured. The image capture settings for COM700 matched those of the "unknown" images in Validation Two (corresponding to setting 1 in Table 4 and Fig. 5). The COM1700 training set was composed of images from both COM700 and COM1000. A "." indicates that no *C. virginica* larvae were present. "**Cumulative accuracy**" was calculated as the total number of true positive classifications for *C. virginica* divided by the total number of *C. virginica* images in all tests combined, multiplied by 100.

		Trained technician		ShellBi software		
Test	Images of *C. virginica*	Goodwin	Wingate	COM700	COM1000	COM1700
A) Validation One						
1	41	98	98	46	93	95
2	1	100	100	0	0	100
3	0	.	.	.	.	.
4	8	100	38	75	75	75
5	20	100	90	65	95	90
6	0	.	.	.	.	.
7	16	100	100	38	94	94
8	19	100	100	74	95	95
9	5	100	80	80	80	80
10	0	.	.	.	.	.
11	16	100	100	100	88	100
12	26	88	96	88	96	96
13	7	100	100	100	86	100
14	10	100	100	80	100	90
15	5	100	100	100	80	100
16	0	.	.	.	.	.
17	19	100	84	95	95	95
18	7	100	100	100	100	86
	Mean	99	92	74	84	93
	Std	3	16	28	24	7
	Cumulative accuracy	**98**	**94**	**73**	**92**	**94**
B) Validation Two						
1	15	100	100	93	60	60
2	44	100	75	84	86	80
3	5	40	80	100	80	100
4	26	100	100	88	96	88
5	5	100	100	100	60	100
6	0	.	.	.	.	.
7	15	100	100	100	47	87
8	37	86	100	100	84	89
9	13	100	100	100	77	92
10	15	100	100	93	93	93
11	35	100	100	86	60	74
12	19	100	100	95	47	79
13	4	100	100	100	25	50
14	16	100	100	81	69	94
15	10	100	100	100	20	50
16	4	100	100	100	50	100

TABLE 6. Continued

Test	Images of *C. virginica*	Trained technician		ShellBi software		
		Goodwin	Wingate	COM700	COM1000	COM1700
17	25	100	100	92	36	60
18	20	100	100	85	25	70
	Mean	96	97	94	60	80
	Std	14	7	7	24	17
	Cumulative accuracy	**97**	**96**	**92**	**65**	**80**

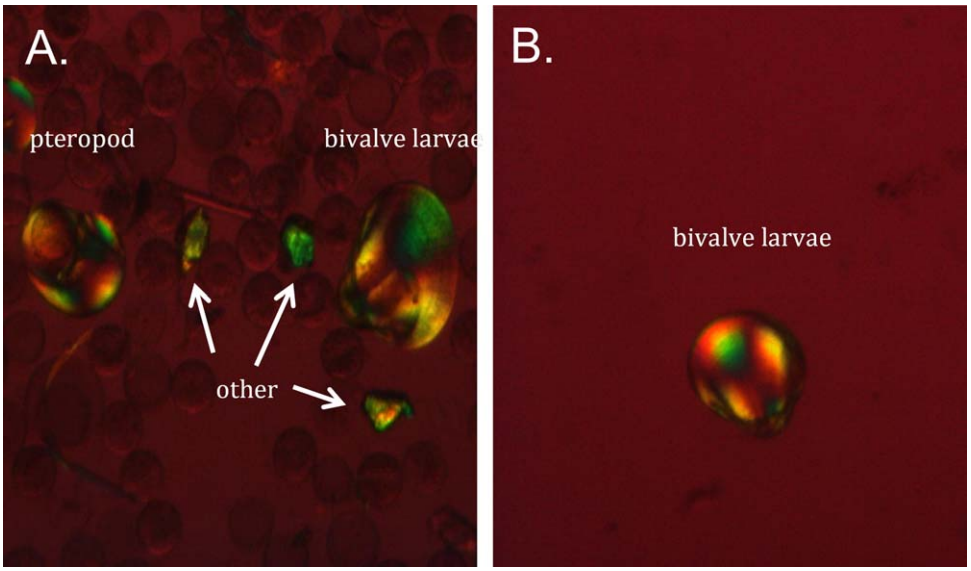

Fig. 6. Images from (A) a field sample and (B) laboratory-reared bivalves which were imaged at 7X magnification. The field sample contained small birefringent materials or other birefringent organisms like pteropods which made it difficult to automate cropping of ROIs.

them with ShellBi software is the fastest way to identify and measure *C. virginica* in the Choptank River to date.

Comments and recommendation

We recommend that protocols be established for maintaining classification accuracy over time, which include: (1) systematic sample preparation, (2) repeated checks of the alignment of the camera and stage (frequently at first (e.g., daily) until a longer period of stability of in alignment is achieved (e.g., weekly), (3) monitoring of color channel light intensity, and (4) repeated classification of a standard.

In keeping with previous assessments of this method (Thompson et al. 2012; Goodwin et al. 2014), we recommend that unknown larvae be imaged using the same microscope and camera settings as those for the training sets. We also recommend that validation studies (similar to those in this manuscript) be conducted when using this technique in different systems and with different species of interest. The validation experiments showed that *C. virginica* larvae could be identified with accuracies $>85\%$ on average for the ShellBi software and $>92\%$ on average for the trained technicians. Therefore, as suggested in Goodwin et al. (2014), the speed of the ShellBi classification technique could be augmented by the high accuracies of a trained technician if a technician checks and corrects the images classified by ShellBi. Because the ShellBi software sorts images into folders for each category of the training set, a trained technician can quickly scan the images to determine if any are out of place.

In addition to imaging bivalve larvae, the automated image acquisition system has application for rapidly acquiring high-magnification images of other planktonic species. For example, the system is being tested for imaging copepods (North and Pierson pers. comm.) and the technique could be used to image pteropods as well (Goodwin, pers. obs.). Images acquired with this system are compatible with image-based software programs designed to identify planktonic organisms, which would increase the user base for the system and its viability as a commercial product. Future work using this system could advance our understanding of

eutrophic and coastal systems around the world by allowing rapid image acquisition and classification of species that require magnification for identification.

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