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Species- and stage-specific barnacle larval distributions obtained from AUV sampling and genetic analysis in Buzzards Bay, Massachusetts, USA

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ABSTRACT

A new method to autonomously collect larvae and environmental variables such as temperature, salinity, and circulation is described. A large volume in situ pumping system, recently developed for discrete biogeochemical sample collection in deep-sea environments, was adapted and mounted to the autonomous underwater vehicle (AUV) REMUS 600 for autonomous larval and environmental sampling in coastal waters. To assess the performance of this system, the distribution of barnacle larvae was assessed in March 2014 with two transects perpendicular to the coastline (~9.9 and 11.2 km) in Buzzards Bay, Massachusetts, USA. The second transect included a complex sampling mission through a relatively deeper channel, and sampling at discrete depth intervals. In this deployment, nearshore and surface waters were fresher, with distinct vertical stratification due to salinity. Barnacle larvae were classified into early nauplii, late nauplii, and cyprid stages, and the mitochondrial COI barcode marker was sequenced to identify individual larvae of different stages. In an analysis of 164 barcode sequences, larvae belonging to *Amphibalanus* sp., *Semibalanus balanoides*, and *Chthamalus fragilis* were found, with *Amphibalanus* sp. the most abundant larval taxon overall. In the second deployment, early and late nauplii were relatively more abundant near the bottom. However, there was no obvious pattern relative to depth with cyprids, and there were no clear cross-shore distributional patterns for nauplii and cyprids. While additional deployments are necessary to corroborate these observations, the results demonstrate the feasibility of this approach. Autonomous vehicle based sampling has the potential to collect larvae of other invertebrates as well as zooplankton, and together with genetic identifications, overcomes many existing limitations and will provide valuable new insight in understanding larval distributions and transport dynamics.

1. Introduction

1.1. Background

Most benthic temperate marine invertebrates have a planktonic larval stage as part of their life cycle, and planktonic larvae are the primary means for dispersal when the adults are sessile or have limited mobility. Larval transport tends to occur during "events" such as internal tidal bores (Pineda, 1999) and upwelling relaxation (Shanks et al., 2000). Larvae are generally poor swimmers and can potentially be carried long distances by ocean currents (Scheltema, 1986). However, many larvae can influence their horizontal distribution by controlling their vertical position (Nelson, 1912; Knights et al., 2006) and thus affect transport and spatial distributions on bottom habitats. Distributional patterns and behaviors are species-specific, but larvae are difficult to identify with traditional methods (Wong et al., 2014; Chen et al., 2013a; Garland and Zimmer, 2002) and knowledge on vertical distribution and behavior is lacking for most species.

There is a crucial need for autonomous larval sampling that can respond to environmental gradients. Adaptive sampling of this kind would greatly facilitate studies of larval transport, a process dependent on larval behavior and physical variability. The ability to do this work, however, is limited by technology and development of testable hypotheses (Pineda et al., 2007). Moreover, fine-scale measurements of larval distribution relative to sharp environmental gradients in temperature, salinity and circulation are needed for elucidating mechanisms of larval transport and to identify sites where larvae accumulate (e.g., fronts).

In particular, water density is a key variable to examine as it drives the stability of the water column, which has a direct effect on the vertical distribution of planktonic organisms (e.g., Ignatiades, 1979, but see Baker and Mann, 2003). An unstratified water column is easily overturned by a short-term wind event, producing a redistribution of the plankton. However, a strongly stratified water column would result in a decoupling of the upper and bottom density layers, and would not be overturned by the same wind event. Moreover, some internal tides and bores transport zooplankton, and the energy of these internal motions is partially dependent on water column stratification (e.g., Pineda and López, 2002).

Traditionally, larvae are sampled with nets or plankton pumps (Chen et al., 2013b; Wiebe and Benfield, 2003), and sampling is performed at

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fixed stations along a predetermined sampling grid. Following collection, larvae are traditionally identified to gross taxonomic level. Invertebrate larvae are difficult or practically impossible to identify to species using morphometric methods (e.g., Wong et al., 2014; Chen et al., 2013a for barnacles), and processing time is often prohibitive with intensive sampling (Garland and Zimmer, 2002). Thus, larvae are often binned into super-specific groupings (e.g., 'bivalves' or 'nauplii') that confound interpretation of results. Overall, current methods for resolving larval distribution are expensive, labor intensive, and inadequate for resolving the episodic and small spatial scales that often characterize larval transport.

Recent advances in engineering and molecular biology overcome methodological limitations and can provide new insights on larval distribution. The Suspended Particulate Rosette (SUPR) multi-sampler was developed by Breier et al. (2009, 2014) to obtain geochemical and microbial samples from the deep-sea. SUPR takes up to 14 sequential discrete samples by in situ filtration and preservation. It has been deployed on a mooring (East Pacific Rise) and in the deep sea using the vehicles *Jason* (Mid-Atlantic Ridge), and *Nereus* (Breier et al., 2009, 2012, 2014). Remote Environmental Monitoring Units (REMUS) 600 vehicle is well suited for mounting SUPR to sample coastal larvae. REMUS swims a programmed track at up to 4.5 knots (8.3 km/h) to 600 m water depth, and at lower speeds with a mission endurance of up to 70 h and range of 286 nautical miles (530 km) (Stokey et al., 2005). REMUS navigates using Doppler Velocity Log assisted inertial navigation which is supplemented with GPS fixes when surfaced and acoustic navigation systems. REMUS uses a propeller and fins for steering and diving, to provide forward speed and control heading and position in the water column.

Additionally, accurate species-specific identification can be achieved using analysis of diagnostic genetic barcodes (Bucklin et al., 2011; Valentini et al., 2009). Typically, genomic DNA from an individual is extracted and a target barcode marker (e.g., the mitochondrial COI gene) is amplified by PCR and sequenced. Sequences are compared to reference sequences from identified individuals for taxonomic assignment. Sequences from individuals within a species are more similar to each other than to sequences originating from other species. In some taxa, such as barnacles, the mitochondrial COI gene may be useful for discriminating species (Wong et al., 2014; Chen et al., 2013a) as well as assessing population genetic differences (Wares and Cunningham, 2001).

1.2. Study system

The focus of this study is on barnacle larvae in Buzzards Bay, Massachusetts, USA. The barnacle *Semibalanus balanoides* (Linnaeus 1767) is the most abundant sessile metazoan in New England rocky shores, and is a model organism for marine ecology, with a representative life cycle and a long history of study. Like most barnacles, *S. balanoides* is hermaphroditic with internal fertilization, and release nauplii into the water column. Larval release occurs in late fall/early winter in Buzzards Bay, in response to winter storms (Gyory et al., 2013). The nauplii feed in the plankton for several weeks, and transition through 6 naupliar stages. At the end of the 6th stage, the nauplii develop into non-feeding cyprid larvae, which settle between January and early May and metamorphose into adults (Pineda et al., 2006). Other barnacles (e.g., *Chthamalus fragilis* (Darwin 1854), *Amphibalanus improvisus* (Darwin 1854), *Amphibalanus eburneus* (Gould 1841) and *Amphibalanus amphitrite* (Darwin 1854)) are also found in the study area and have similar reproductive strategies and overlapping planktonic larval periods (Lang and Ackenhusen-Johns, 1981) although their timing of reproduction is less well studied.

1.3. Objectives

The goal of this study was to develop an autonomous larval sampling capability coupled with genetic analysis to study larval distribution. The performance of our method for describing the vertical and cross-shore distribution of larvae was assessed. Here, SUPR was coupled to REMUS and deployed in Buzzards Bay when barnacle larvae are known to be abundant. Barnacle larvae were enumerated and staged, and a fraction was identified using DNA barcoding. Species-specific and stage-specific distribution patterns with depth were recorded. We evaluate the potential utility of our approach based on our results.

2. Methods

2.1. Autonomous sampling

SUPR was mounted in the forward payload section of the REMUS 600 AUV (Fig. 1A, B). The payload section consisted of two end rings connected with struts. The SUPR components (pump, valve, filter holders, and electronics, Fig. 1A) were mounted to the struts. The SUPR junction box was redesigned to fit within the hull diameter of the AUV. Foam and weights were added to match the AUV diameter and provide the proper buoyancy and trim. The inlet for SUPR was placed outside the hull at the head of the AUV, and the seawater intake opening was ~0.7 cm diameter. Power and two-way RS-232 communication were provided to SUPR in a single underwater cable connection from a REMUS AUV guest port. Custom-developed software integrated REMUS and SUPR and enabled sample collection at preplanned locations within the dive track.

2.2. Field deployments

Two deployments in Buzzards Bay, Massachusetts were undertaken in March, 2014 using the coastal research vessel R/V Tioga (Fig. 1B). Barnacle larvae are usually abundant at this time (Blythe and Pineda, 2009; Pineda et al., 2002, 2006). The first deployment, on March 14, 2014, was a relatively short trial deployment and the second deployment, on March 24, consisted of a longer and more complex sampling mission (Fig. 2). The first and second deployment sampling patterns were approximately cross-shore, and whereas the first was approximately linear (9.9 km), the

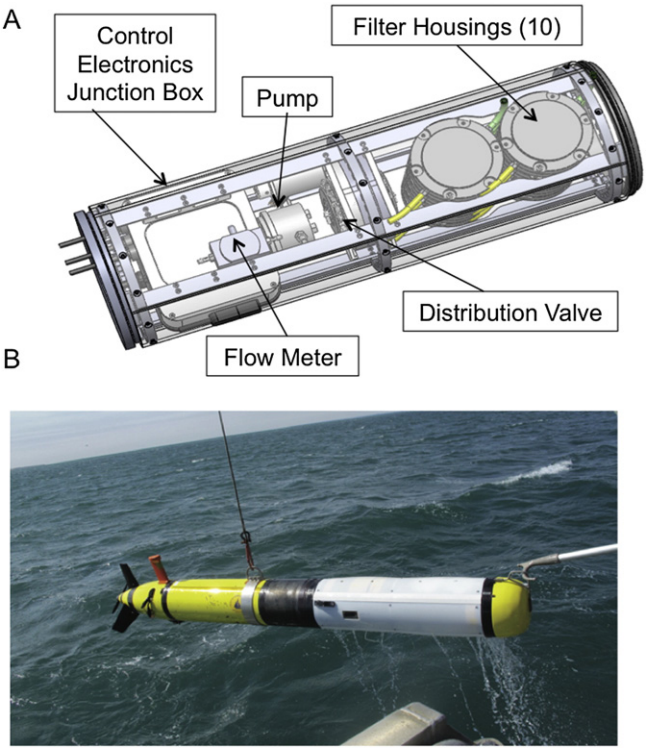

Fig. 1. (A) Schematic of SUPR components installed on REMUS 600. (B). SUPR–REMUS being recovered from the research vessel R/V Tioga.

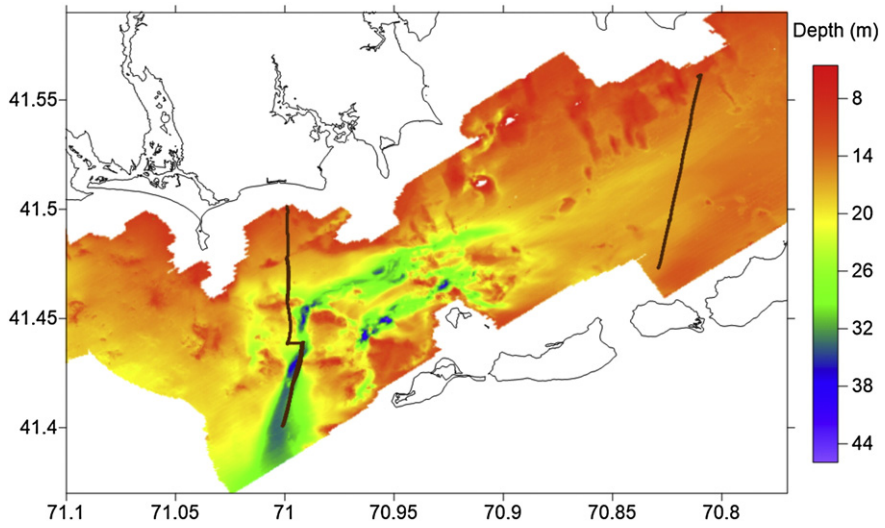

Fig. 2. SUPR–REMUS tracks in Buzzards Bay, MA. The track on the right is from the March 14 trial deployment. The track on the left is from the March 24 deployments. Bathymetric data from Ackerman et al. (2012).

second followed a longer, angled pattern to resolve a complex bottom channel (total linear distance covered 11.2 km). The SUPR sampler is programmed to start to operate at the beginning of the mission and while operating, water is pumped through each filter at predetermined times for a fixed amount of time. Sampling missions were programmed in advance and included discrete sampling at different depth zones for the second deployment. The AUV followed a sawtooth vertical pattern ("yo-yo") that in the first deployment extended from near the surface to near the bottom, approximately 15 m depth (Fig. 3A). In the second deployment, the upper boundary of each sampling interval was relative to the surface (depth) while the lower boundary was relative to the bottom (altitude) (Fig. 3B), with maximum depths down to ~32.3 m. SUPR filters were fitted with 200 μm mesh, which retain barnacle larvae as well as other invertebrate larvae and zooplankton. Flow rate was recorded using a digital flow meter situated adjacent to the pump (Fig. 1A). SUPR did a three minute purge between samples, where water remaining in the tubing was expelled, to prevent contamination between samples. Meshes and retained plankton were preserved in 95% ethanol immediately upon return to the laboratory. REMUS was equipped with a CT sensor (Neil Brown Ocean Sensors, Inc., NBOSI G-CTD) to obtain conductivity and temperature measurements and a pressure sensor (Paroscientific) to obtain depth. Sampling rate was ~5 Hz. Data from the CT sensor were used to derive density.

2.3. Larval analysis

Barnacle larvae were enumerated and nauplii staged using a dissecting microscope. For cyprids and larger nauplii species identification, DNA was extracted using DNEasy Blood & Tissue extraction kits (Qiagen). For early stage and some later stage larvae, individuals were placed directly in the PCR tube, omitting the extraction step (Chen et al., 2013a). The mitochondrial COI gene was amplified by PCR using either the standard HCO and LCO primers (Folmer et al., 1994) or the jgHCO (Geller et al., 2013) and mInt primers (Leray et al., 2013), which amplify a shorter but still diagnostic region of mtCOI that is internal to the Folmer primer amplicons. For amplifications using the Folmer primer set, the PCR cycle was: a) an initial denaturation at 95° for 3 min; b) 35 cycles of 95° 30 s, 48° 30 s, 72° 60 s; and c) a final extension at 72° for 5 min. For the Geller–Leray primer set, a touchdown protocol was used (Leray et al., 2013). When necessary to obtain sufficient DNA for sequencing, the Geller–Leray primer set was used in a secondary amplification using the touchdown protocol with the Folmer PCR product. PCR products were visualized on a 1.5% agarose gel stained with GelRed

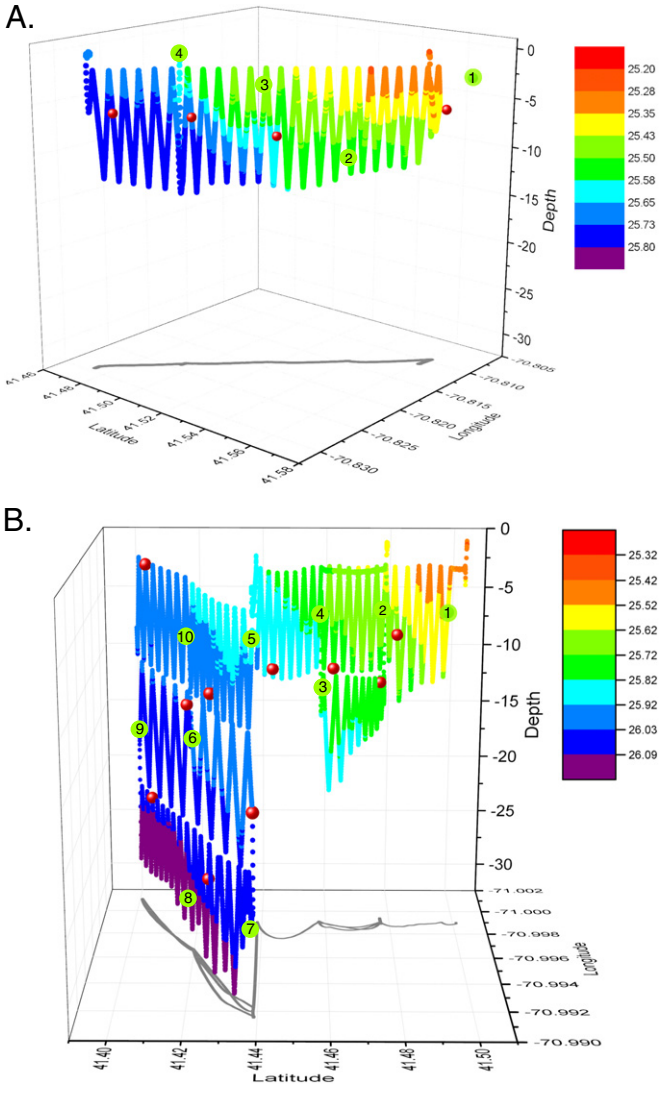

Fig. 3. (A) Track lines from the March 14 deployment. (B) Track lines from the March 24 deployment. Numbers designate samples, and the gray lines on the bottom are a latitude–longitude projection of the track. Color indicates density (σ_t). Density data is not available for the first sample in the March 14 deployment.

(Biotium), and purified with Qiaquick PCR Purification kits (Qiagen). Purified products were quantified using a Nanodrop 2000 spectrophotometer and sequenced in both directions (Eurofins MWG Operon). Chromatograms were assembled and confirmed by eye using Geneious 7.1.7 (Biomatters Limited) and aligned using ClustalW (Larkin et al., 2007), with default parameters. To assign our larval barcodes to species, local barnacle adults were sequenced following the above protocols and relevant mtCOI sequences from GenBank were downloaded to use as references (*A. amphitrite*: KC138445; *A. improvisus*: FJ845841; *S. balanoides*: FJ84815; *C. fragilis*: AF234813). These sequences were incorporated into the alignment and included in subsequent analyses. The alignment was trimmed to remove regions of missing and poor quality data from the ends and sites with ambiguous base calls. Sequence data was collapsed into haplotypes for ease of visualization. Taxonomic assignment was determined by generating neighbor-joining haplotype trees based on Kimura 2-parameter distances using PAUP* 4.0b10 (Swofford, 2003). Support for nodes was estimated from 1000 bootstrap replicates. Larvae were categorized into groupings defined by date (first or second deployment) and stage (cyprid, late nauplii stages V and VI, early nauplii stages I–IV). All larval haplotypes and adult reference sequences generated in this study were deposited in GenBank (KM649630–KM649670).

3. Results

3.1. Deployments

The first deployment on 14 March 2014 coincided with a period of *S. balanoides* cyprid settlement, just after the seasonal high (Govindarajan and Pineda, unpublished data; Pineda et al., 2006). We obtained four samples (Fig. 3A). Flow rates for all samples averaged 2.93 ± 0.132 l/min. The first sample filtered for 11 min, and the other three samples filtered for 21 min. The first sample filtered 0.031 m^3 of seawater and the other three samples filtered 0.060–0.066 m^3 of seawater. The water column was cold (1.1–1.5 °C), and there was little temperature variability with depth (<0.2 °C within any station along the transect). Waters near the landmass were slightly colder than waters more to the south of the Bay (~0.3 °C difference for the upper 10 m). Surface waters near the landmass were slightly fresher than offshore waters (~0.2 PSU difference), and waters near the landmass (31.4 PSU for the upper 10 m) were fresher than offshore waters (31.9 PSU for the upper 10 m) through the entire water column. Waters near the landmass, to the north of the transect, were lighter than offshore waters (Fig. 3A).

The second deployment on 24 March 2014 undertook a more complex sampling mission. The first part of the mission ran southward through relatively shallow water (to 20 m), then shifted to the east, and continued toward the southwest through a deeper channel at the mouth of Buzzards Bay. We obtained ten samples along this transect (Fig. 3B). The first sample was in relatively shallow water, and the entire water column was sampled. Samples 2 and 3 covered the surface and deeper portions of the next position along the cross-shore transect. Sample 4 included surface water only, and sampled through an active shipping later. Deeper sampling was not attempted in this location in order to avoid potential interactions with vessels. The transect then turned into deeper water and two more offshore areas were sampled, each with a surface, middle, and deeper layers (samples 5, 6, and 7 and samples 10, 9, and 8). For each of the 10 samples, SUPR filtered for 17 min. Flow rates averaged 3.035 ± 0.146 l/min, and the volume filtered was 0.048–0.056 m^3 per sample. During the second deployment the entire water column was warmer than during the first (~1.3 °C difference for the upper 10 m), and water temperature ranged from 2.6 to 2.9 °C. No clear cross-shore patterns in temperature were found. The water column was slightly saltier than in the first deployment, and salinity ranged from 31.9 to 32.3 PSU, with fresher water near the landmass than offshore. Similar to the first deployment, waters near the landmass were lighter than offshore waters (Fig. 3B).

3.2. Larval abundance and identification

Barnacle larvae were abundant in both dates, with densities up to ~950 larvae/m^3 (Fig. 4). Early stage nauplii (i.e., stages I–IV) tended to be more abundant than later (stages V and VI) nauplii and cyprids. 58 larval barcodes were obtained from the first deployment and 106 larval barcodes were obtained from the second deployment (Fig. 5). Attempts to amplify and sequence some larvae, particularly early stage larvae that were processed relatively later after sampling, failed, possibly due to degradation of the small amount of larval tissue. Most cyprids were sequenced, although those from sample 3 in the second deployment were

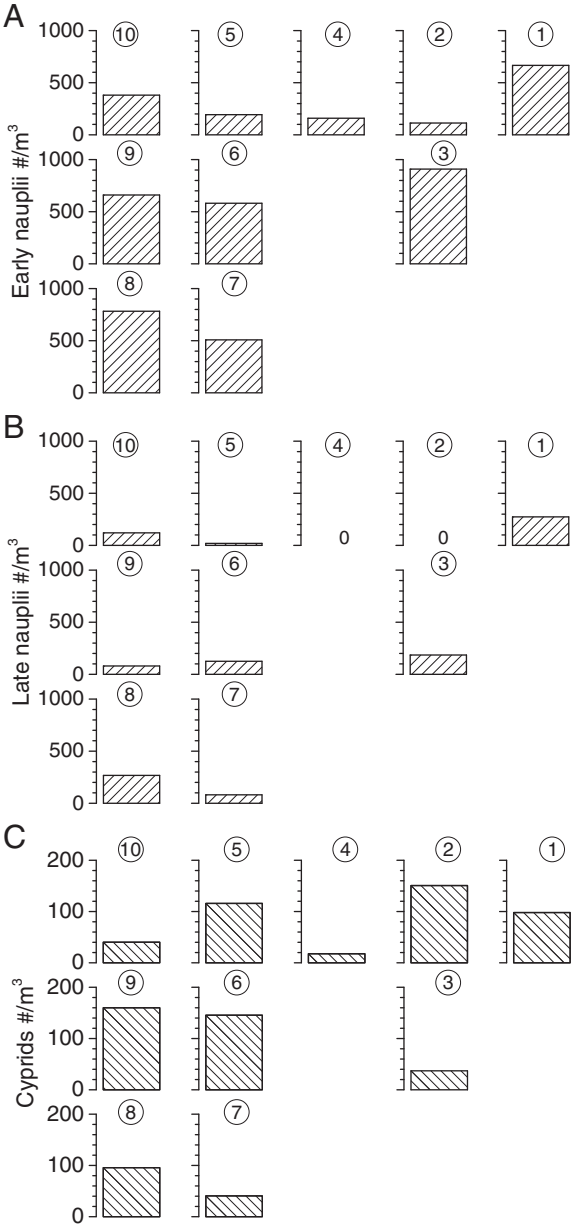

Fig. 4. (A) Early stage nauplii; (B) late-stage nauplii; and (C) cyprid concentrations for the second (March 24, 2014) deployment. The vertical scale is the same for early and late nauplii, but smaller for the cyprids reflecting their lower concentration. Numbers near bars correspond to sample numbers in Fig. 3. Histogram arrangement reflects sampling order relative to the sea surface: histograms in the top row of each panel reflect the surface layer sampling; histograms in the middle reflect mid water column sampling; and histograms at the bottom of each panel sampled near the bottom. Sample 1 was in shallow water and so collected larvae through the entire water column (surface to bottom). Sample 4 collected larvae in the surface layer and sampling was not conducted in the middle or bottom layers at this location.

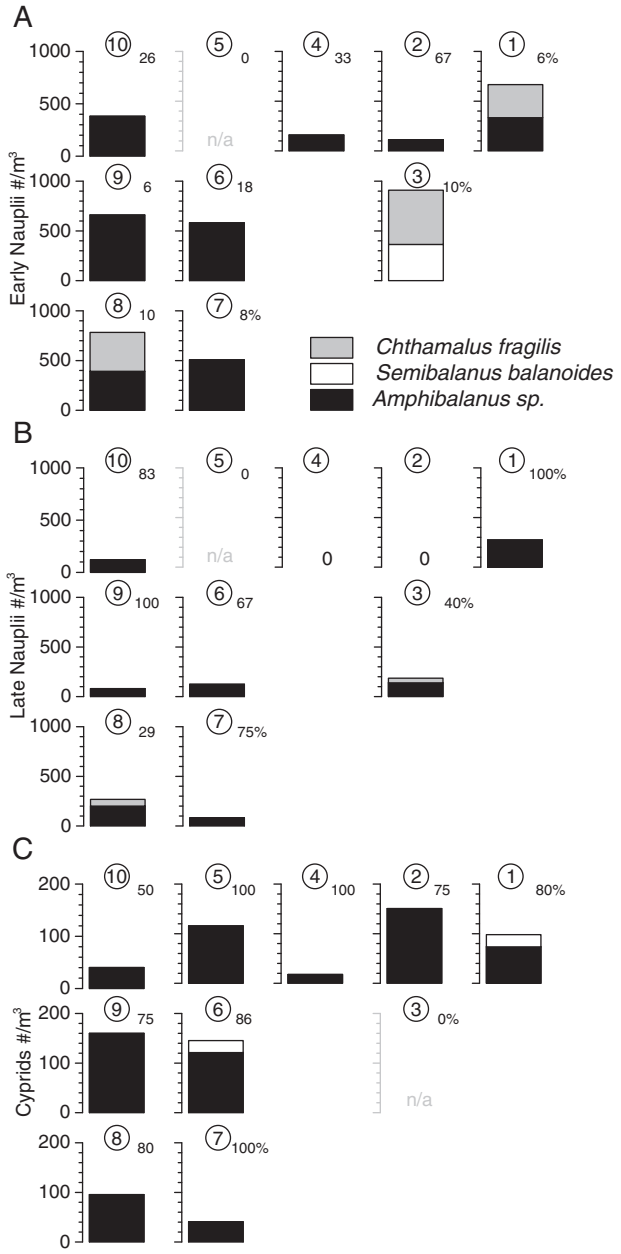

Fig. 5. (A) Early stage nauplii; (B) late-stage nauplii; and (C) cyprid species composition based on barcode data in Buzzards Bay, 24 March 2014. Numbers near bars correspond to sample numbers in Fig. 3 and arrangement of histograms is the same as in Fig. 4 . Shading indicates the proportion of barcode sequences for a given species and assumes that the number of barcodes represents the percent composition of that species in the entire sample. Proportion of successfully barcoded larvae indicated above the bars. No late nauplii were found in samples 2 and 4 and no cyprids were sequenced in sample 3 due to a procedural error.

not due to a procedural error. The trimmed alignment for the first deployment consisted of 270 base pairs with 94 variable sites and 16 larval haplotypes. The trimmed alignment for the second deployment consisted of 268 base pairs with 96 variable sites and 23 larval haplotypes. All barcode sequences fell into monophyletic clades that also included a reference sequence with 100% bootstrap support (Fig. 6A, B). Larvae of *S. balanoides*, *Amphibalanus* sp., and *C. fragilis* were found in samples from both deployments. *Amphibalanus* sp. was the most abundant barnacle species, and several early nauplii, late nauplii, and cyprids were found in samples from both deployments. There was no match for our *Amphibalanus* sp. sequences on GenBank, but it matched sequences from *Amphibalanus* sp. adults collected subtidally in Buzzards Bay off of Woods Hole, Massachusetts. Attempts to identify morphologically *Amphibalanus* sp. failed, in part because very few individuals could be found in the field.

Species and stage specific larval composition differed between the two March surveys (Fig. 5). *Amphibalanus* sp. nauplii and cyprids were found in all Deployment 1 samples and it was the most abundant species in samples 2, 3, and 4. In sample 4, it was the only species collected. All stages were found in all samples, except no late nauplii were found in sample 1. *S. balanoides* was most abundant in sample 1 but also present in samples 2 and 3. *C. fragilis* were found in sample 2 (one early stage nauplius and 3 cyprids).

An even greater proportion of sequenced larvae belonged to *Amphibalanus* sp. in Deployment 2. A single *S. balanoides* cyprid was

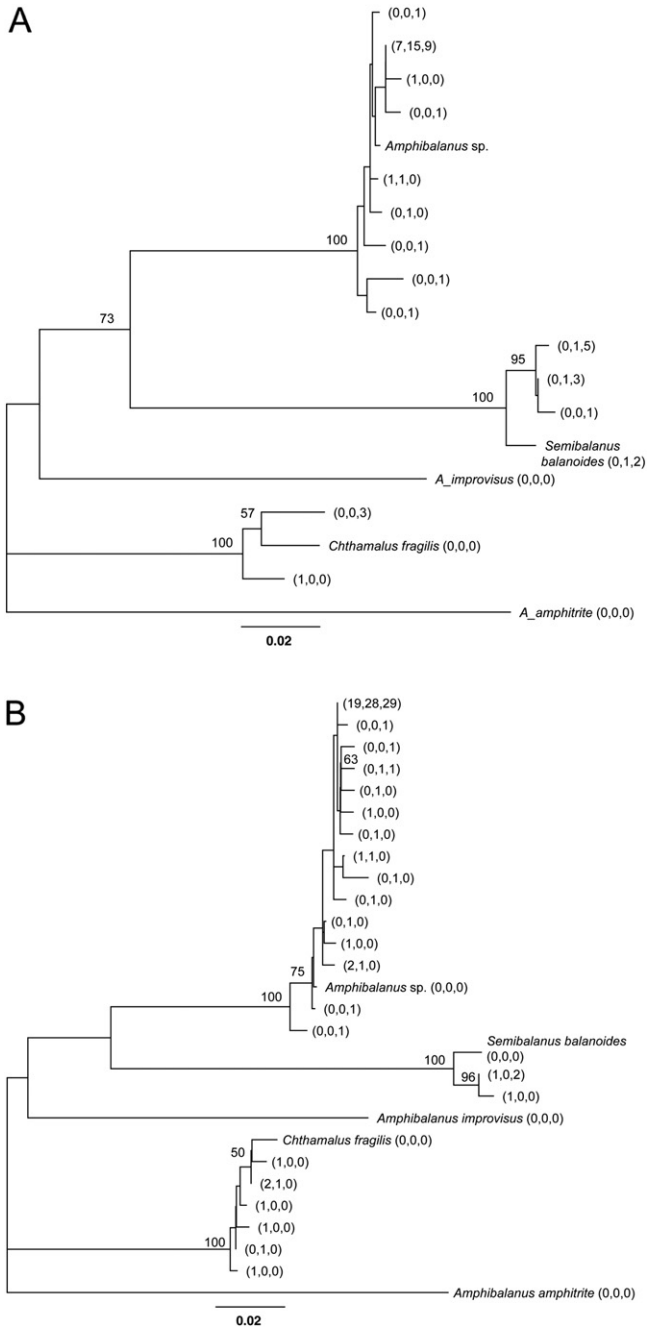

Fig. 6. (A) Neighbor joining tree of larval haplotypes (unique DNA sequences) from the first deployment. (B) Neighbor-joining tree of larval haplotypes from the second deployment. Numbers in parenthesis at the tips of the branches refer to the number of early nauplii, late nauplii, and cyprids, respectively, sharing that haplotype.

found each in the fractions of sample 1 and sample 6 that were sequenced. Two early stage *S. balanoides* nauplii were found in sample 3. A small number of early and late stage *C. fragilis* nauplii were found in both samples 3 and 8.

3.3. Depth and cross-shore distribution

Relatively fewer early stage nauplii occurred in the surface samples (samples 2, 4, 5, and 10) than in bottom samples (samples 1, 3, 7, and 8) (Fig. 4A). Although the overall number of individuals was much smaller, this general pattern was also found in late stage nauplii (Fig. 4B). Relatively fewer larvae were found in the surface, above-bottom samples (samples 2, 4, 5, and 10) than in bottom samples (samples 1, 3, 7, and 8). For cyprids, there was no clear pattern relative to depth (Fig. 4C). When broken down further by species, *Amphibalanus* sp. early nauplii were found at all depths while *S. balanoides* and *C. fragilis* nauplii tended to be found only in bottom samples (samples 1, 3, and 8; Fig. 5A). *Amphibalanus* sp. late nauplii tended to occur in near bottom and midwater samples (Fig. 5B). None were found in two of the surface samples (samples 2 and 4). *C. fragilis* late nauplii were only observed in the near bottom sample 8, and no *S. balanoides* late nauplii were detected (Fig. 5B). No clear pattern with depth was observed for *Amphibalanus* sp. cyprids, and *S. balanoides* cyprids were only found in samples 1 and 6 and none in samples that covered only surface waters (Fig. 5C). No *C. fragilis* cyprids were detected. With respect to cross-shore distribution, there are no clear patterns for the most abundant taxon, *Amphibalanus* sp., or for the other less abundant species, *S. balanoides* and *C. fragilis*.

4. Discussion

4.1. Autonomous sampling

Coupling advances in robotics and genetics can allow the testing of marine ecological hypotheses that are difficult to investigate using traditional methodology (Ryan et al., 2014 and Harvey et al., 2012). The vertical distribution of larvae in the water column influences larval transport, but traditional sampling methods do not provide sufficient resolution or sample effectively near the bottom beyond shallow bottom depths. The Continuous Plankton Recorder (CPR) has provided a wealth of plankton distribution data over several decades. However it samples at a constant ~10 m depth and so does not address vertical distributions (Reid et al., 2003). The Multiple Opening and Closing Net, with an Environmental Sensing System (MOCNESS) and similar systems can take samples over discrete depth intervals and is coupled with environmental sensors (Wiebe, 1976; Wiebe et al., 1985), but can only be used in open waters, above the bottom. Moored plankton pumps can provide depth-specific information if they are deployed in an array (Garland et al., 2002), but only sample at a fixed point. Shipboard deployed plankton pumps can be used to sample in layers, but simultaneously sampling along a transect, or sampling very close to the bottom in deep waters, is practically impossible (Tapia et al., 2010). AUVs have the flexibility to sample over different intervals, including 10s of cm above bottom and are accompanied by environmental sensors. Harvey et al. (2012) and Ryan et al. (2014) used the AUV *Dorado* to sample plankton in Monterey Bay, California, although their sample volumes were smaller. The *Dorado* "gulps" 1.8 l seawater into collection bottles in contrast to SUPR, which is based on filtration of variable water volumes and in the deployments in this study filtered between 30 and 66 l per sample. Still even these volumes are small for larval samples and it is possible that as configured, SUPR–REMUS may miss taxa that are dilute or with effective escape behaviors. Future improvements can incorporate modifications to increase flow rate as well as the development of adaptive sampling capabilities. Our methods can also be adapted for different target taxa, by changing the size of the mesh openings; for example, finer mesh may be substituted to sample smaller invertebrate larvae such as bivalves.

4.2. Genetically-identified larvae

Morphological identification of invertebrate larvae can be extremely difficult or practically impossible. Many larval taxa are not well described, there may be few taxonomically relevant features, and features change over the course of larval development. Even when larval descriptions are available, identification is laborious and time-consuming. Genetic barcoding permits accurate species-level identifications, although the results presented here demonstrate the importance of having a comprehensive reference database (Webb et al., 2006). The lack of a positive match on GenBank for our most abundant larval taxon indicates that additional sequencing of identified individuals is necessary, even for well-studied coastal areas like Buzzards Bay. While *Amphibalanus* sp. sequences could not be matched with an identified GenBank reference, Lang and Ackenhusen-Johns (1981) observed larvae from *Amphibalanus balanus*, *A. crenatus*, and *A. improvisus* in late winter/early spring. However, we were unable to identify morphologically the adults that corresponded to our larval sequences. The likely existence of cryptic species in many taxa including barnacles (Wares, 2001) further supports the need for additional sampling across the entire potential range of nominal species.

The mtCOI sequences clearly distinguished the different larval taxa, even though only a relatively short portion of the gene was used. These results suggest that the Leray–Geller mtCOI primer combination can be useful in next-generation barcode sequencing of barnacles, which produces shorter read lengths than the conventional Sanger sequencing technology used here (Shokralla et al., 2012).

4.3. Larval timing

C. fragilis nauplii were unexpectedly found in mid-March, during the first deployment, although they are typically found in warmer late spring and summer months. McDougall (1943) observed *C. fragilis* settlement from June to November in Beaufort, NC and inferred larval release in early to mid-May. Fish (1925) observed nauplii in Woods Hole in July and August. Similarly, Lang and Ackenhusen-Johns (1981) observed *C. fragilis* nauplii from May through August in nearby Rhode Island waters, and inferred a restricted breeding season in mid-summer. These studies are also consistent with our observations on *C. fragilis* settlement in Buzzards Bay during late summer (Pineda, unpublished data). The presence of *C. fragilis* cyprids in mid-March suggests that either the reproductive season is considerably longer or the larvae originate from an unexplained source. It is unlikely that *C. fragilis* nauplii overwintered from the previous year, given the length of time that the larvae would have to survive in harsh conditions. However, this possibility has been considered for other taxa although it has not been tested rigorously. Mann (1985) hypothesized overwintering of larvae of the bivalve *Arctica islandica* to explain their unexpected presence in the plankton early in the season. Conway et al. (1990) postulated that larvae of the barnacle *Lepas pectinata* could survive in deeper water with relatively lower energetic cost, to extend the larval period.

4.4. Vertical and cross-shore distribution

The few studies that have examined the vertical distribution of barnacle larvae found that patterns appear to be species-specific, and these results are consistent with these findings. In the second deployment, when most of the larvae were *Amphibalanus* sp., nauplii were found at all depths, although they were generally more abundant in middle and near-bottom samples. There was no clear pattern with depth for *Amphibalanus* sp. cyprids. Tapia et al. (2010) study in southern California found cyprids concentrated closer to the bottom, while nauplii were more abundant at the surface. Dos Santos et al. (2007) sampled

the vertical distribution of cyprids off northwest Portugal. At least one barnacle species appeared to exhibit diel vertical migration, occurring in surface waters at night and at depth during the day. Other species were found only in the surface waters. Garland et al. (2002) and Rawlinson et al. (2005) also found evidence of barnacle vertical migration, but no evidence of vertical migration was found in southern California barnacle larvae (Tapia et al., 2010). SUPR–REMUS sampling in both deployments occurred during the day only, so vertical migration was not examined.

Whereas ocean waters near the landmass were fresher and less dense than offshore waters, no cross-shore patterns in barnacle larvae distribution were detected. Stage-specific patterns in cross-shore distribution of barnacle larvae, such as those found by Tapia and Pineda (2007) for two species of Southern California intertidal barnacles might be restricted to open coastlines, as opposed to bays with multiple sources of larvae from opposite facing shores. Moreover, it is not clear whether *Amphibalanus* sp. is a nearshore species or not, and cross-shore larval distribution might be partially influenced by adult habitat.

4.5. Conclusions

To overcome current limitations in larval sampling methodology, the AUV REMUS 600 was fitted with the SUPR pump to obtain discrete samples of barnacle larvae along sawtooth transects and DNA barcoding was used to discriminate species. SUPR–REMUS was successfully deployed twice in March, 2014. *Amphibalanus* sp. was the most common larval species and was abundant in samples from both deployments. *S. balanoides* and, unexpectedly, *C. fragilis* larvae were also present in the first deployment.

The primary objective of this research was to test a new methodology for sampling larvae, so caution must be taken in interpreting ecological conclusions from a single, unreplicated transect. Additionally, although the volume of water that was sampled was larger than other autonomous methods under development (Ryan et al., 2014; Harvey et al., 2012), it is relatively small compared to what can be sampled in net tows and limits the robustness of any conclusions. While the ecological conclusions are limited, the results obtained demonstrate the feasibility of an autonomous approach coupled with molecular identification, and highlight areas to concentrate future efforts.

SUPR–REMUS has several advantages over existing methods in terms of the flexibility to perform complex sampling missions and near-bottom sampling. Coupled with genetic analysis, SUPR–REMUS is an effective new method to characterize invertebrate larval distributions. Future studies will include development to increase flow rates to sample organisms that are more dilute or have active escape behaviors. Furthermore, adaptive sampling relative to environmental parameters will be incorporated to test hypotheses of larval transport.

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