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Direct setting of *Crassostrea virginica* larvae in a tidal tributary: applications for shellfish restoration and aquaculture

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ABSTRACT: Recent efforts to restore eastern oyster *Crassostrea virginica* populations in Chesapeake Bay have targeted regions where low larval supply limits recruitment. A common practice in areas of minimal spat set incorporates remote setting, a method of setting hatchery-reared larvae in tanks, and then transporting them to the field. Although remote setting is effective, inefficiencies exist. Repeated shell handling, spat mortality during transport, and decreased cultch supply suggest that other restoration methods merit consideration. We present the results of the first known field test of directly seeding a submerged oyster reef with larvae and setting an additional cohort on the reef the following year. We surrounded a 65 m^2 reef located in 2.5 m of water with a flexible enclosure and added 2.3×10^6 larvae. Larvae were allowed 3 d to set on either clean or fouled shell valves, after which we removed the enclosure. Setting efficiencies in the enclosure (spat produced per larvae introduced), were 26 and 7 % for clean and fouled shell, respectively, in 2012, and 10 and 16 % in 2013. These are comparable to published remote setting efficiencies, and were significantly higher than in our representative shoreside tank . Spat densities on site 1 mo post-set (median = 189 spat m^{-2}) and the following spring (115 juveniles m^{-2}) met best practice restoration metrics. Larvae were re-set on the reef in 2013, but in lower densities than 2012, supplementing the reef with 23 spat m^{-2}. In areas of minimal natural recruitment, or low cultch availability, direct setting of larvae in the field may be a viable alternative to remote setting.

KEY WORDS: Oyster · Restoration · Aquaculture · Remote setting · Larvae · Methods

INTRODUCTION

Populations of the eastern oyster *Crassostrea virginica* Gmelin have fallen to <1 % of historic levels in Chesapeake Bay (Newell 1988, Wilberg et al. 2011) due to natural and anthropogenic stressors such as overharvesting, diseases, sedimentation, and eutrophication leading to hypoxia and anoxia (Baker & Mann 1992, Rothschild et al. 1994, Paynter 2007). Additionally, changes in freshwater flow (Volety 2008, Pollack et al. 2011), increased predation (O'Beirn et al. 2000), reduced resistance to parasites (Lenihan et al. 1999), and lowered food availability (Osman et al. 1989, Zajac et al. 1989, Lenihan et al. 1999) can further lower growth within and recruitment to already stressed stocks (Kimmel & Newell 2007). The collapse of the *C. virginica* population has damaged the economy and ecosystem of Chesapeake Bay (Kirkley & Lipton 1995, Ford & Tripp 1996). Increasing *C. virginica* populations may both

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augment harvests and provide ecosystem services such as improved water quality (Cerco & Noel 2007, Fulford et al. 2007, Grizzle et al. 2008), increased nutrient assimilation and denitrification (Kellogg et al. 2013), and increased macrofaunal habitat (Peterson et al. 2003, Rodney & Paynter 2006). For these reasons, efforts over the last decade have been made to restore oyster populations not only in Chesapeake Bay, but world-wide (Paynter 2007, Schulte et al. 2009, Beck et al. 2011, Kennedy et al. 2011).

In areas of low natural recruitment, such as mesohaline tributaries, a primary method of seeding oyster beds to both restore ecosystem services and enhance aquaculture relies on remote setting of larvae (Bohn et al. 1995, Congrove et al. 2009). In this technique, pediveliger-stage larvae are purchased from a hatchery, transported to the desired site, and set on cultch (generally whole *C. virginica* shell contained in bags or cages) in shoreside tanks. After 2 to 3 d, water within the tanks is exchanged, and larvae are fed via a flow-through seawater system that may be supplemented with algae (Wallace et al. 2008). Two to 10 d post-set, the new spat-on-shell are transported to a nursery site, generally in shallow or intertidal waters (Supan 1991, Congrove 2008). After the spat reach the size of 2 cm, they are moved by small boat or barge to deeper reefs for restoration or bottom leases for aquaculture (Supan 1991, Bohn et al. 1995).

The remote setting method has proven effective for oyster production along US Atlantic, Pacific, and Gulf of Mexico coasts (Jones & Jones 1988, Supan 1991, Congrove 2008, Ippolito 2008). Setting larvae in tanks offers the advantages of controlling water temperature, salinity, and dissolved oxygen during set, and potentially reduces access of predators to the spat (Webster & Meritt 1985, Bohn et al. 1995). Additionally, light aeration of the water allows for more even distribution of larvae and deeper penetration into the cultch, increasing set (Webster & Meritt 1985, Supan 1991).

While effective, the technique requires extensive materials handling. Spat set on cultch in tanks must be moved to the nursery and then transported once again to the final grow-out location, with each successive move likely adding some degree of oyster mortality. As the standard *C. virginica* shell cultch is becoming less available (Powell et al. 2006, Mann et al. 2009), other substrata such as marl rock, clam shell, porcelain, and cobble may need to be used (Nestlerode et al. 2007, Chesapeake Bay Foundation 2010, George et al. 2014), likely incurring a greater material handling cost.

A method that may expand reef-seeding capacity while minimizing cultch handling is setting of oyster larvae onto reefs *in situ*, or 'direct setting'. There is precedence for this method of restoration in a number of different benthic invertebrate taxa including corals (Heyward et al. 2002) and bay scallops *Argopecten irradians* (Arnold 2008, Leverone et al. 2010). Direct setting has been used to set *C. virginica* on small plots (<10 m^2) in shallow or protected waters (Burke 2010, Steppe et al. 2010, Rahall et al. 2011, Theuerkauf et al. 2015), but has not been demonstrated over larger areas (e.g. >50 m^2) or in deeper water (>2.0 m), conditions which are more relevant to larger-scale restoration or aquaculture. Direct setting may also facilitate adding additional cohorts of oysters to restored reefs or aquaculture leases without incurring the cost of providing additional substratum. As *C. virginica* is considered protandronous, several year classes are essential to produce mixed-gender reefs, and thus multiple age cohorts are a desired metric for 'successful' oyster restoration (United States Army Corps of Engineers 2012, Baggett et al. 2014).

The goal of this study was to test the direct setting method in a tidal tributary by enclosing a reef with a flexible structure and adding *C. virginica* larvae within the enclosure. Specifically, the study was designed to determine whether initial setting efficiencies (spat set per larvae added) and recruitment produced by directly setting larvae in the field was comparable to that produced via the established shoreside remote setting method. An additional objective was to compare set on clean shell base (representing a newly emplaced reef) with set on fouled shell base (representing substrata emplaced between 1 mo and 1 yr ago) to quantify how effective direct setting would be in adding year classes to previously constructed restoration or bottom harvest aquaculture sites.

MATERIALS AND METHODS

Study area

The experiment was conducted in St. Leonard Creek, a tidal tributary of the Patuxent River Estuary, on Maryland Commercial Aquaculture Lease #602 (CA602, leaseholder: The Johnny Oysterseed Company; 38.3929°N, 76.48864°W, Fig. 1). Previous water quality data collected at Morgan State University Patuxent Environmental and Aquatic Research Lab (MSU-PEARL) showed temperature, salinity,

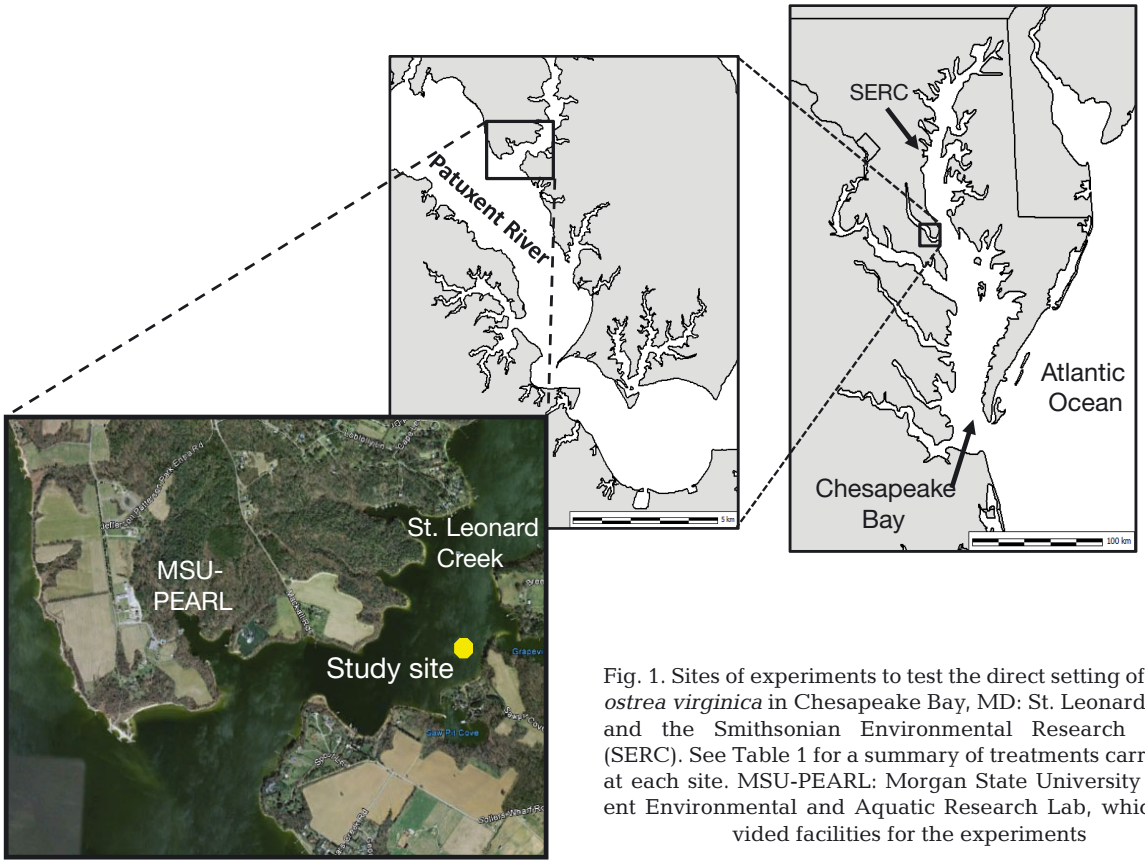

Fig. 1. Sites of experiments to test the direct setting of *Crassostrea virginica* in Chesapeake Bay, MD: St. Leonard Creek and the Smithsonian Environmental Research Center (SERC). See Table 1 for a summary of treatments carried out at each site. MSU-PEARL: Morgan State University Patuxent Environmental and Aquatic Research Lab, which provided facilities for the experiments

and dissolved oxygen to generally be favorable for *Crassostrea virginica* larval set (Bohn et al. 1995). Water depth at the study site ranges from 2.0 to 3.0 m, and hydrodynamics are driven by tides, local winds, and recreational boat traffic. Permits were obtained from Maryland Department of the Environment (General Tidal Wetlands License 12-PR-1070), the US Army Corps of Engineers (CENAB-OP-RMN2102-61047-M07), and the United States Coast Guard (USCG), and the study site was marked by USCG approved buoys and marker lights to deter recreational boat traffic.

Larval enclosure design and mooring trial

In order to set the engineering design constraints for the larval enclosure and mooring system, waves and currents were measured with an acoustic wave and current meter (AWAC) at 2 high-energy locations adjacent to CA602 in 2012. Hydrodynamics at CA602 were verified in 2013 with an additional 1 mo AWAC deployment in late spring. The enclosure consisted of a Type-III PVC silt barrier curtain (Granite Environmental), typically deployed in marine construction, and similar in composition to sediment curtains previously used to directly set shellfish larvae in shallow waters (Leverone et al. 2010, Theuerkauf et al. 2015). Two 15.2 × 4.6 m sections of curtain were purchased and joined with American Society for Testing Materials (ASTM) standard aluminum connectors and lacing through grommet holes at the end of each section. Furling lines were used to shorten parts of the enclosure so that it reached the substratum throughout the aquaculture lease, yet did not produce extra drag in the shallower areas. A 0.3 m float maintained the top of the enclosure at the surface, while a 0.95 cm diameter steel ballast chain encased in the curtain sleeve ensured that the bottom of the enclosure was flush with the substratum (Fig. 2). Further details of the structure construction and deployment are provided in Fredriksson et al. (2016).

An initial trial of the stability of the enclosure was performed from 31 August to 4 September 2012, immediately prior to the larval setting experiment. At that time the enclosure was towed to the study site and moored with four 10 kg Danforth anchors at apex locations and tensioned with a line between oppos-

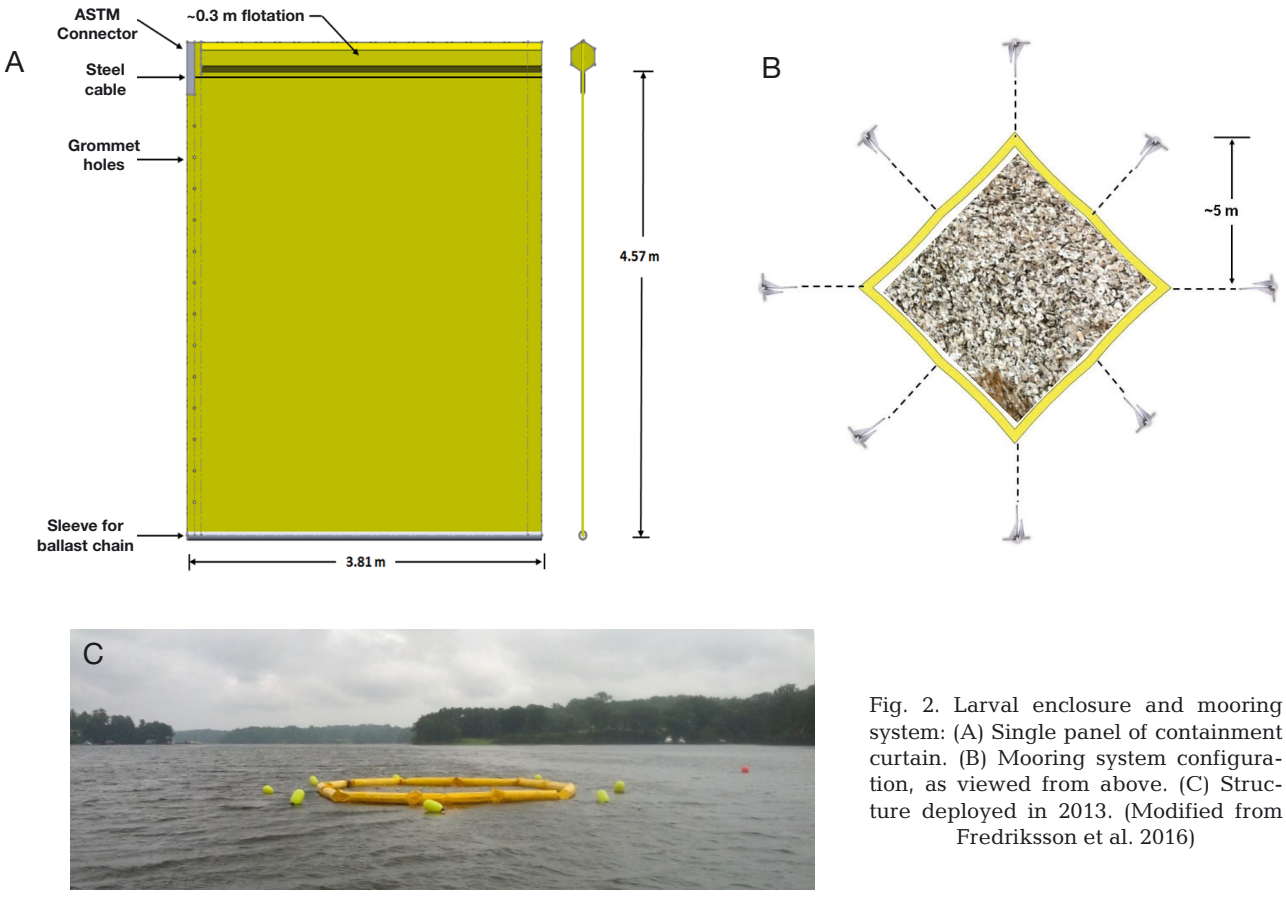

Fig. 2. Larval enclosure and mooring system: (A) Single panel of containment curtain. (B) Mooring system configuration, as viewed from above. (C) Structure deployed in 2013. (Modified from Fredriksson et al. 2016)

ing sides. Four additional 6 kg Danforth mid-span anchors were then set, forming an octagonal enclosure that surrounded an area of approximately 65 m^2 (Fig. 2). The owner of CA602 then placed 5.88 m^3 (128 bushels) of clean aged oyster shell valves inside the enclosure leaving a base layer approximately 0.09 m thick. Because adding the aged shell base temporarily increased turbidity, 2 sections of the curtain were pulled up for approximately 18 h, after which the sides were lowered completely. Yellow Springs Incorporated Model 6600 sondes were placed both inside and outside the enclosure to measure water quality every 15 min during the stability and setting trials (Fig. 3).

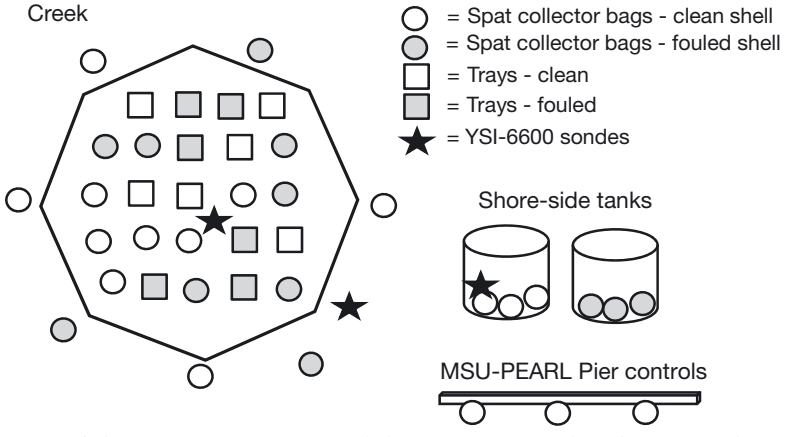

Fig. 3. Schematic representation of the treatments and replicates used in the 2012 larval set experiments. In 2013, clean and fouled spat collector bags were combined in a single tank

Larval setting trials

The larval setting experiment included assessments of initial set in shoreside tanks and the field enclosure, as well as survival after 1 mo and overwinter. Conditions of clean and fouled shell base, and setting larvae on the emplaced reef in a second year were used to determine whether the method could be beneficial for re-seeding existing reefs and aquaculture leases. To represent standard remote setting techniques, two 208 l cylindrical tanks (0.56 m diameter × 0.91 m height) were filled with ambient creek water and

placed in a shoreside raceway at MSU-PEARL to prevent the tanks from over-heating during the study. The 0.25m^2 bottom of each tank was lined with 125 clean oyster shell valves, producing a layer of shells roughly 0.04 m thick. Air stones were added to reduce the risk of hypoxia during larval set, and a water quality sonde was placed in 1 tank to monitor temperature, salinity, and dissolved oxygen every 15 min for the duration of the experiment.

Approximately 3.55×10^6 competent eyed oyster *C. virginica* Gmelin larvae were obtained from the University of Maryland Center for Environmental Sciences Horn Point Hatchery in Cambridge, Maryland, and transported for 4 h by car to MSU-PEARL. The larvae were held in a damp paper towel enclosed in a plastic container and cooled with paper-wrapped ice packs. Larvae were refrigerated at approximately 1.6°C for 4 d, after which the plastic container containing the larvae was removed from the refrigerator and placed in a bucket containing 10 l of creek water (temperature = 28°C, salinity = 13.7). After 30 min the larvae were removed from the container and added directly to the bucket of creek water. Larval concentrations were assessed by pipetting four 1 ml samples of the larvae/water mixture onto a Sedgwick-Rafter slide. It was anticipated that each slide would contain 355 larvae, (a 1 ml sample of 3.55×10^6 larvae added to a 10 l bucket). Though not all were actively swimming, the majority of larvae counted appeared to be alive. Examination of the samples indicated that each slide contained (mean ± SD) 234 ± 32 larvae ml^{-1}. It was assumed that any larvae not counted in the samples had either already set in the original bucket or had experienced mortality in transit from the hatchery, and were therefore not observed swimming in the water column. This suggested that of the larvae purchased, 66% were available for set, somewhat higher than previously published estimates of survival from hatcheries (Bohn et al. 1995).

The contents of the initial bucket were then divided into 4 buckets, each containing 2.5 l of the larvae/water mixture. To each of these buckets, 7.5 l of creek water was added so that each container held 10 l of the larvae/water mixture. The contents of each bucket were stirred frequently to reduce setting on the sides and bottom of the container. Of the total larvae, 0.5% were set aside for each of the shoreside control tanks. It is noted that the ratio of larvae added to the tank to those added to the enclosure was 1:197, yet the ratio of the bottom area of the tank to the bottom area of the enclosure was 1:285. This disparity was expected to lead to relatively higher setting efficiencies in the shoreside tanks than in the enclosure, but the ratios were scaled as closely as was tractable while minimizing the risk of larvae setting prematurely during transport to the study site.

The experimental design, including all replicates, treatments, and sampling times are illustrated in Table 1. Treatments simulating the biofouling community representative of previously emplaced reefs had been prepared 1 mo prior to the start of the experiment by deploying 3 mesh bags (1.5 cm mesh, 0.03 m^3) of clean, aged oyster shucking house shell valves from the MSU-PEARL pier. By early September, visual inspection indicated that the shell valves were covered with a microbial biofilm and a macroinvertebrate community that included the bryozoan *Victorella pavida*, amphipods (*Corophium* sp., and *Gammarus* sp.), clam worms (*Nereis* sp.), and mud worms *Polydora lingi*. No *C. virginica* spat large enough to be seen without a microscope were observed on the fouled shell treatments.

Three sample spat collector bags (55 × 20 cm, 1.5 cm diameter mesh containing 20 oyster shell valves) were added to one of the shoreside tanks, and 3 spat collector bags containing fouled shell valves were placed in the second tank. It is recognized that separating clean and fouled shell conditions did not replicate conditions in the enclosure, so when the experiment was repeated in 2013, clean and fouled shell bags were placed in a single shoreside tank. Approximately 12 000 larvae were added to each tank to scale the larvae/bottom area ratio as closely as possible to the field setting enclosure. The remaining buckets of larvae were combined, placed in a 208 l carboy, which was transported 10 min by boat to the study site within the creek.

Previously, 21 spat collector bags containing either clean or fouled shell valves had been prepared for field deployment: 6 clean and 6 fouled bags for inside the enclosure; 3 clean and 3 fouled for outside the enclosure; and 3 clean bags for pier controls (Fig. 3). Twelve plastic sample trays (32 × 24 × 15 cm), each containing 30 valves (6 with clean and 6 with fouled valves) were also placed in the enclosure (Table 1). It was assumed that larvae would initially set comparably on valves in both trays and mesh bags, but the open sample trays would reduce the risk of restricted water flow due to bio-fouling as the spat continued to grow on site. Due to the restricted dimensions of the shoreside tanks, tray samples were not included in the tank conditions.

Shell condition (clean versus fouled valves) samples were assigned to locations in the enclosure by dividing the enclosure into a grid and using a com-

Table 1. Experimental design to test the direct setting of eastern oyster *Crassostrea virginica* larvae in Chesapeake Bay. (A) Initial set, measured 3 d after larvae were added to the enclosure. (B) 1 mo post-set. SLC: St. Leonard Creek (on-site); SERC: Smithsonian Environmental Research Center raceway (off-site); MSU: Morgan State University. Unshaded treatments showed sets approximating 0.0 spat per shell (SPS), and were therefore not included in the statistical analysis. NA: not applicable

A. Initial set

Year	Location	Spat collector type	Shell type in collectors	Number of spat collectors placed at location	Number of clean valves examined	Number of fouled valves examined
2012	Enclosure	Tray	Clean and fouled	12 (6 of each)	30 (5 tray^{-1})	28 (4 or 5 tray^{-1})
	Enclosure	Bag	Clean and fouled	12 (6 of each)	None	None
	Tank 1	Bag	Clean	3	15 (5 bag^{-1})	NA
	Tank 2	Bag	Fouled	3	NA	15 (5 bag^{-1})
	Surrounding enclosure	Bag	Clean and fouled	6 (3 of each)	None	None
	MSU pier	Bag	Clean	3	None	NA
2013	Enclosure	Tray	Clean and fouled	12 (6 of each)	30 (5 tray^{-1})	30 (5 tray^{-1})
	Enclosure	Bag	Clean and fouled	12 (6 of each)	None	None
	Tank 1	Bag	Clean and fouled	6 (3 of each)	15 (5 bag^{-1})	15 (5 bag^{-1})
	Surrounding enclosure	Bag	Clean and fouled	6 (3 of each)	None	None
	MSU pier	Bag	Clean	3	None	NA

B. One month post-set

Year	Initial set location	Grow-out location	Spat collector type	Shell type in collectors	Number of spat collectors	Number of clean valves examined	Number of fouled valves examined
2012	Enclosure	SLC	Tray	Clean and fouled	12 (6 of each)	120 (20 tray^{-1})	120 (20 tray^{-1})
	Enclosure	SERC	Bag	Clean and fouled	12 (6 of each)	120 (20 bag^{-1})	120 (17–20 bag^{-1})
	Tank 1	SERC	Bag	Clean	2	29 (14–15 bag^{-1})	NA
	Tank 2	SERC	Bag	Fouled	3	NA	44 (14–15 bag^{-1})
	Enclosure	SLC	Quadrat	Clean	6 quadrats	Varied	NA
	Surrounding enclosure	SLC	Quadrat	Fouled	6 quadrats	NA	Varied
	Surrounding enclosure	SERC	Bag	Clean and fouled	6 (3 of each)	60 (20 bag^{-1})	60 (20 bag^{-1})
	MSU pier	SERC	Bag	Clean	3	60 (20 bag^{-1})	NA
2013	Enclosure	SLC	Tray	Clean and fouled	12 (6 of each)	120 (20 tray^{-1})	117 (18–20 tray^{-1})
	Enclosure	SERC	Bag	Clean and fouled	12 (6 of each)	116 (16–20 bag^{-1})	15 (15–20 bag^{-1})
	Tank 1	SERC	Bag	Clean and fouled	6 (3 of each)	60 (20 bag^{-1})	54 (15–20 bag^{-1})
	Enclosure	SLC	Quadrat	Fouled	6 quadrats	NA	Varied
	Surrounding enclosure	SLC	Quadrat	Fouled	6 quadrats	NA	Varied
	Surrounding enclosure	SLC	Bag	Clean and fouled	6 (3 of each)	120 (20 bag^{-1})	120 (20 bag^{-1})
	MSU pier	SLC	Bag	Clean	3	60 (20 bag^{-1})	NA
	SERC raceway	SERC	Bag	Clean	3	60 (20 bag^{-1})	NA

puter random number generator to position the bags and trays. Four rows of 6 sample trays or bags were placed in the enclosure by hand-lowering using lines attached to small floats. This resulted in a completely randomized, yet not blocked design. The remaining spat collector bags were then placed outside the enclosure to test for larval leakage, and 3 spat collector bags were hung beneath the MSU-PEARL pier, 1400 m from the study site, to assess potential natural spatfall during the study.

Prior to adding the larvae, the water within the enclosure was exchanged by pulling up 2 segments of the structure and lowering them again after 15 min. The larvae were released in small batches using a pitcher attached to a long boat hook, as a small boat moved around the perimeter. The enclosure was left in place to allow the larvae to set (Fig. 2).

After 3 d all treatments were recovered, and the enclosure and mooring system were removed from the study site. At that time, the first 5 shell valves of approximately equal size were selected from each of the 12 trays. Due to the difficulty observing the newly-set spat, even with at 50 to 150× magnification (Nikon SMZ1500), only live spat on the insides of the valves were counted. The trays were then replaced in the creek at the location at which the enclosure had been deployed.

Initial set was next quantified on 5 subsampled valves from each of the shoreside tank spat collector bags. Then, to represent early transport to a nursery site and to facilitate post-set measurements, all the spat collector bags were transported in aerated 19 l buckets to an off-site grow-out raceway at the Smithsonian Environmental Research Center (SERC) in Edgewater, Maryland adjacent to the Rhode River. Water temperatures and salinities of the Rhode River and the Patuxent River fall within similar ranges during the summer (Maryland Department of Resources, www.eyesonthebay.net), though water flow, and therefore food supply, was likely lower at the SERC off-site raceway than in St. Leonard Creek (Grizzle et al. 2008). While some spat mortality was expected during transport (Jones & Jones 1988), it was assumed that transport would affect all treatments equally, so that relative survival among treatments could still be compared.

One month survival, overwinter mortality, and adding a second cohort

Approximately 1 mo post-set, the on-site trays in St. Leonard Creek were recovered by divers. Spat per shell was quantified on the first 20 valves of approximately equal size haphazardly subsampled from each tray. To differentiate between spat set during the study versus natural set that may have occurred in the creek after the experiment, measurements of shell height and length were taken from the first 20 spat found per tray, with no more than 3 spat measured per shell valve. Because no spat substantially smaller than the mean (i.e. more than 2 standard deviations from the mean) were observed in the trays, it was unlikely that additional set took place. To quantify total set and size of spat on the reef itself, divers excavated the shell base (to the anoxic layer) from 6 quadrats (243× 360 mm) haphazardly selected along 2 perpendicular transects that crossed at the center of the reef. Six quadrats located off the reef were also sampled to test for larval 'leakage' from the site. Total spat that set within each quadrat excavated were then counted. All spat values were converted to spat m^{-2} to allow for comparison with published oyster restoration metrics (Baggett et al. 2014). All trays and shell base were then returned to the reef.

Spat survival in the collector bags placed in the off-site raceway was also analyzed 1 mo after initial set. Although spat survival and growth after 1 mo likely differed from that at the study site, it was assumed that the relative survival among treatments in the raceway would not differ from survival at the study site.

Overwinter mortality of the 2012 cohort of oysters was measured prior to setting new larvae on the reef in 2013. In early May 2013, divers repeated the protocol used for the 1 mo post-set sampling the previous fall, recovering the trays left on site and excavating shell base. No quadrats were sampled off the reef because spat set between October and May is uncommon in Chesapeake Bay (Kennedy1996). Spat per shell in the 12 trays was determined, and 10 spat were selected haphazardly from each tray for dimension measurements. Density of juvenile oysters m^{-2} were compared to the October 2012 spat m^{-2} values to determine overwinter mortality.

To assess the potential of adding a second year class to an existing reef, new competent oyster larvae purchased from the University of Maryland's Horn Point Hatchery were set at the study site in July 2013. The procedure followed in 2013 was identical to that in 2012, with the exceptions that the experiment was run approximately 7 wk earlier in the summer; and clean and fouled shell treatments were contained in a single shoreside tank, better replicating conditions in the field enclosure. The larval enclosure was trans-

ported to the site on a small barge and deployed using the GPS coordinates of the enclosure in 2012. Initial spat set was quantified as in the previous year, though in 2013 set on both sides of the shell valves was counted, and 2 small panels (~6 × 6 cm) were examined for spat set on the enclosure material itself. Analysis of samples grown out for 1 mo on and off site occurred in early August 2013. Because the focus of this study was the survival of young-of-the year juvenile oysters, only spat set in 2013 were counted in the quadrats.

Statistical analysis

In 2012, counts of initial spat set were limited to the inside of shell valves. Therefore, to compare set between years, and to quantify spat mortality from initial set to the following month, initial counts from 2012 were multiplied by 1.4. This value was obtained from the ratios of larvae that set on both sides of the valves versus inside of the valves in 2013, i.e. 1.4:1.0. Some variation in set may have occurred due to position of the samples in the enclosure, yet the random assignment of each sample tray and collector bag was assumed to reduce the effect of position within the enclosure. Spat set was minimal or absent on spat collector bags deployed outside the enclosure (<0.001 spats per shell valve, hereafter SPS), on the pier control bags (0.0 SPS), and on the panels that formed the larval enclosure (0.0 SPS), so these treatment conditions were removed from further statistical analyses.

Prior to analysis, counts of SPS were $\ln(x + 1)$ transformed to better meet the assumption of normality. Within the enclosure and within the tanks, the effect of shell type (clean vs. fouled shell base) was tested with a mixed model nested ANOVA, with shell type as a fixed factor, and sample (tray) as a random factor nested within shell type (Sokal & Rohlf 1995). This procedure was repeated for the 1 mo off-site sample bags, and on-site trays. Because standard transformations did not improve normality or equality of variances, spat densities inside the enclosure were compared to those outside the enclosure using a Wilcoxon rank sum test, the nonparametric equivalent of a 2-sample *t*-test (Sokal & Rohlf 1995).

Setting efficiency (spat produced vs. larvae added) in the enclosure was compared to the shoreside tank, though it is noted that the experimental design in 2013, when clean and fouled treatments were co-located in a single tank, more closely represented the conditions within the enclosure. Maximum values of spat per shell were calculated (Table 2) based on the number of larvae added to the enclosure and tank each year and assuming that there are 12 000 oyster valves in a cubic meter (given there are 550 valves in a market bushel, Meritt &Webster 2014) and all larvae could access the entire 0.09 m shell layer (Congrove 2008). Setting efficiency was determined by taking the back-transformed means of SPS in clean or fouled shell treatments during initial set, and comparing those values to maximum possible spat set. Setting efficiencies in the enclosure and tank were compared using an approximate test for equal proportions (Ramsey & Schafer 2002). Water quality data were analyzed by examining ranges, means and standard deviations of the parameters. All tests were performed in MATLAB 2015b (Mathworks) and significant differences among treatments were defined at the level of $p = 0.05$.

RESULTS

Water quality

During the larval setting period in 2012, salinity was favorable for *Crassostrea virginica* set (Bohn et al. 1995). Salinity varied with semidiurnal tidal flow outside the enclosure (13.6 ± 0.2, n = 290), yet was slightly higher, and remained relatively stable inside the enclosure (13.8 ± 0.06, n = 290; Fig. 4). In 2013 the sonde within the enclosure did not function, yet salinity outside the enclosure of 10.3 ± 0.2 (n = 590)

Table 2. Calculations of setting efficiency in experiments to test the direct setting of *Crassostrea virginica*. Larval survival from the hatchery was calculated from serial dilutions prior to sets. Maximum possible spat per shell (SPS) was calculated assuming 5.88 m^3 of shell were in the enclosure, each of which contained approximately 12 000 valves

Year	Larvae purchased	Larval survival (%)	Larvae added	Location	Shell type	Maximum possible initial SPS
2012	3.55×10^6	66	2 319 570	Enclosure	Clean Fouled	33
			11 715	Tank	Clean Fouled	63
2013	3.55×10^6	61	2 143 845	Enclosure	Clean Fouled	30
			10 828	Tank	Clean Fouled	44

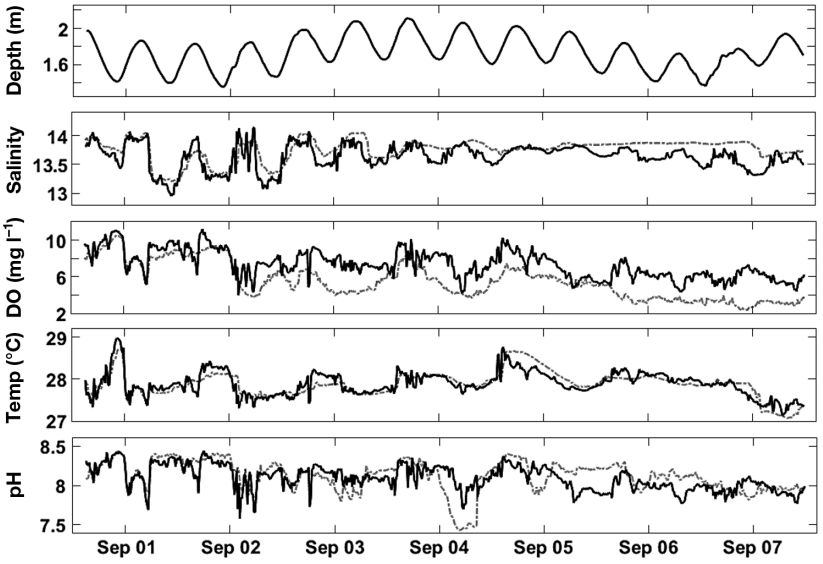

Fig. 4. Water depth at the study site in 2012 and water quality inside (gray dashed lines) and outside the enclosure (black lines) during initial trials (Aug 31 to Sep 4) and larval set (Sep 4 to Sep 7) during the 2012 experiment. While dissolved oxygen (DO) in the enclosure remained above 2.0 mg l^{-1} for the entire study period, the low levels during the end of the trial suggest a need for mesh water-exchange portals in the enclosure. Note that within the enclosure, salinity was less affected by tidal action than outside the enclosure

suggested favorable conditions existed within the structure.

Dissolved oxygen and pH cycled daily both within and outside the larval enclosure. However, reduced exchange with creek water and high biological oxygen demand during the larval setting period caused oxygen to drop more rapidly within the enclosure than in the creek (Fig. 4). Although dissolved oxygen fell to ~35% saturation (2.6 mg l^{-1}) at the end of the setting trial in 2012, it only remained low for a few hours. Short-term exposure to 35% saturation has not been shown to reduce *C. virginica* pediveliger survival, set, or spat survival (Widdows et al. 1989), and so it was assumed that low oxygen did not reduce set in this study. Dissolved oxygen levels in 2013 were greater than observed outside the enclosure in 2012, suggesting that the larvae experienced sufficient oxygen levels in 2013. Typical of shallow estuaries (Tyler et al. 2009), pH cycles of 0.5 occurred daily inside and outside the enclosure.

Initial set

In 2012, initial spat set did not vary significantly between clean (mean = 8.4 SPS) and fouled (2.4 SPS) shell conditions in the enclosure, but set was significantly higher on clean valves (4.6 SPS) than fouled valves (0.6 SPS) in the tank (Table 3A, Fig. 5). Set of new cohort of larvae in 2013 was comparable to 2012, yet did not differ between

Table 3. Comparison of initial set in experiments to test the direct setting of *Crassostrea virginica*. (A) Spat per shell (SPS) in clean versus fouled shell treatments. The null hypothesis tested was that the mean SPS on clean shell base was equal to the mean SPS on fouled shell base (H_0: $\mu_{clean} = \mu_{fouled}$). To correct for non-normality, raw counts were $\ln(x + 1)$ transformed prior to analysis. Adjusted *F*-statistics and p-values account for the random factor of sample within each condition, and were obtained with a mixed model ANOVA incorporating sample as a random factor nested within shell type (clean vs. fouled). $\bar{X}^{\star}$ indicates a back-transformed mean. (B) Setting efficiency in the enclosure versus the shoreside tank. The null hypothesis tested was that the proportion ($\hat{p}$) of larvae successfully setting in the enclosure was equal to the proportion setting in the tank (H_0: $\hat{p}_{enclosure} = \hat{p}_{tank}$). Note that clean and fouled shells were set in separate tanks in 2012 but in the same tanks in 2013. Significant p-values are shown in **bold** type

A. Clean vs. fouled shell treatments

Year	Location	$\bar{X}^{\star}_{SPS}$ clean	$\bar{X}^{\star}_{SPS}$ fouled	*F*	p	Adjusted *F*	Adjusted p
2012	Enclosure	8.4	2.4	10.72	**0.002**	4.23	0.066
2012	Tank	4.6	0.6	13.66	**<0.001**	8.60	**0.042**
2013	Enclosure	3.1	4.9	2.66	0.109	1.68	0.225
2013	Tank	2.3	1.7	0.45	0.506	0.31	0.610

B. Enclosure vs. shoreside tanks

Year	Shell type	$\hat{p}_{enclosure}$	$\hat{p}_{tank}$	*z*	p
2012	Clean	0.26	0.07	129.96	**<0.001**
2012	Fouled	0.07	0.01	42.92	**<0.001**
2013	Clean	0.10	0.05	13.02	**<0.001**
2013	Fouled	0.16	0.04	7.545	**<0.001**

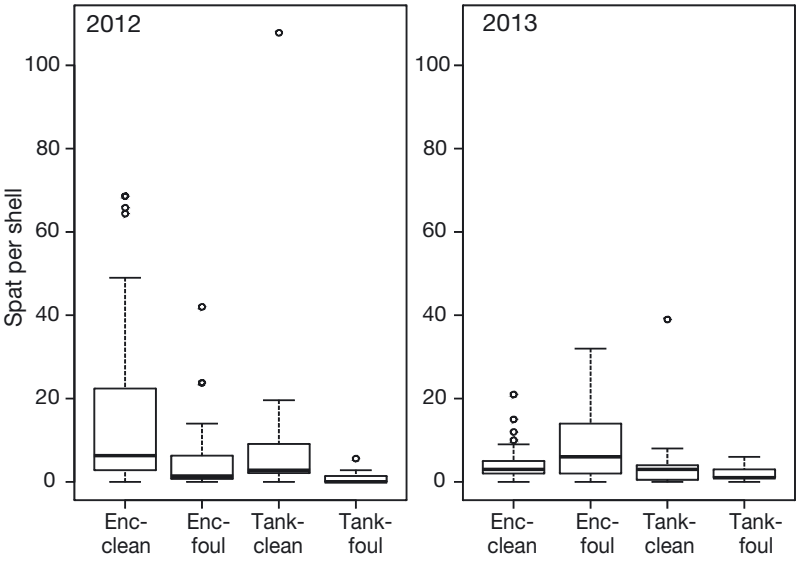

Fig. 5. Initial set of larvae in 2012 and 2013 in treatments with clean and fouled shells in the enclosure and in the shoreside tank. Bold lines show medians, boxes enclose 2nd and 3rd quantiles, and bars indicate 5th to 95th percentiles. Open circles designate outliers.. In 2012, there was no difference in set between clean and fouled shell conditions in the enclosure, but set was significantly higher on clean shells than fouled ones in the tank (mixed model nested ANOVA, p = 0.042). No significant differences were observed between set on clean or fouled shells in 2013 (mixed model nested ANOVA, p = 0.225)

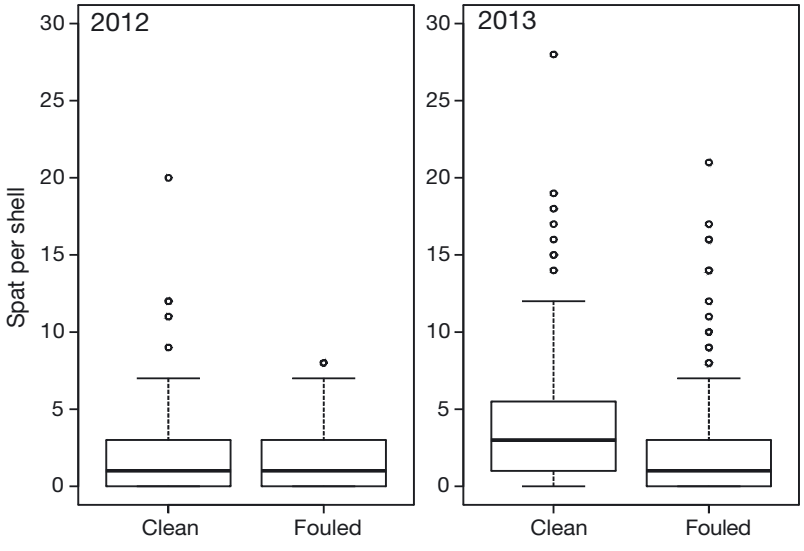

Fig. 6. Spat per shell (SPS) in on-site trays 1 mo post-set. See Fig. 5 legend for explanation of box and whisker plots. No significant difference in SPS occurred between clean and fouled shell treatments after 1 mo in either 2012 (p = 0.468) or 2013 (p = 0.060)

clean and fouled shells in the enclosure (mean = 3.1 and 4.9 SPS, respectively; p = 0.225), or in the shoreside tanks (mean = 2.3 and 1.7 SPS, p = 0.610; Table 3A, Fig. 5).

Setting efficiencies in the enclosure were 26% for clean base in 2012; 7% for fouled shell in 2012; 10% for clean shell in 2013; and 16% for fouled shell in 2013 (Table 3B). Over the course of the study, this led to average setting efficiencies in the enclosure of 18 and 12% for clean and fouled shell base, respectively. Setting efficiencies in the shoreside tanks were 7 and 1% for clean and fouled shell in 2012, and 5 and 4% for clean and fouled shell in 2013, with averages of 6 and 2.5% for clean and fouled shell over the 2 sets. Applying a test of proportions to these values, setting efficiency in the enclosure was significantly higher than in the shoreside tank for both clean and fouled shell conditions ($p < 0.001$ in both years, Table 3B).

One month survival, overwinter mortality, and adding a second cohort

In 2012, 1 mo following set, mean SPS for clean and fouled valves in on-site trays was 1.3 and 1.0, respectively, illustrating no effect of shell type (p = 0.468; Fig. 6, Table 4A). These values translate to survival rates of 15% for spat set on clean shell base, and 42% on fouled shell base. Similar results were obtained in 2013, with no significant difference occurring between SPS for clean (mean = 2.9) or fouled shell valves (mean = 1.4) (p = 0.060; Table 4A, Fig 6).

After 1 mo SPS no longer varied among any set location or shell conditions in samples moved to the off-site raceway. In 2012, mean SPS was 0.6, 0.7, 0.7, and 0.5 in the clean enclosure, fouled enclosure, and clean and fouled tank treatments, respectively (mixed model nested ANOVA, p = 0.944; Table 4B, Fig. 7), corresponding to survival rates of 68, 14, 22, and 6% respectively. A similar pattern was seen in 2013, with SPS values in the 4 treatments of 2.1, 0.7, 0.5, and 0.1, respectively (mixed model nested ANOVA, p = 0.212; Table 4B, Fig. 7), and survival rates of 7, 29, 15, and 83%, respectively, over the 1 mo period.

In 2012, direct sampling of the emplaced reef showed that spat densities were significantly higher

Table 4. Comparison of spat per shell (SPS) in clean vs. fouled shell treatments 1 mo post-set in experiments to test the direct setting of *Crassostrea virginica*. (A) On-site in the enclosure at St. Leonard Creek: clean versus fouled shells. The null hypothesis tested was that SPS on clean shell base was equal to SPS on fouled shell base (H_0: $\mu_{clean} = \mu_{fouled}$). To correct for non-normality, raw counts were $\ln(x + 1)$ transformed prior to analysis. Adjusted *F*-statistics and p-values account for the random factor of sample within each condition, and were obtained with a mixed model ANOVA incorporating sample as a random factor nested within shell type (clean vs. fouled). (B) Off-site at the Smithsonian Environmental Research Center (SERC) raceway: comparison of SPS among the 4 larval setting treatments, i.e. on clean and fouled shells in the enclosure, and on clean and fouled shells in the shoreside tanks. The null hypothesis tested was that there was no difference in mean SPS among the treatments of clean shell base in the enclosure, clean shell base in the tanks, fouled shell base in the enclosure, and fouled shell base in the tanks (H_0: $\mu_{clean\ enclosure} = \mu_{clean\ tank} = \mu_{fouled\ enclosure} = \mu_{fouled\ tank}$). *F*-statistics and p-values reflect results from a multiway mixed model ANOVA with the random factor of 'sample', nested within the fixed factor of 'treatment'. $\bar{X}^{\star}$ indicates a back-transformed mean. Significant p-values are shown in **bold** type

A. On-site

Year	$\bar{X}^{\star}_{SPS}$ clean	$\bar{X}^{\star}_{SPS}$ fouled	*F*	p	Adjusted *F*	Adjusted p
2012	1.3	1.0	1.94	0.165	0.57	0.468
2013	2.9	1.4	19.26	**<0.001**	4.51	0.060

B. Off-site

Year	$\bar{X}^{\star}_{SPS}$ clean enclosure	$\bar{X}^{\star}_{SPS}$ fouled enclosure	$\bar{X}^{\star}_{SPS}$ clean tank	$\bar{X}^{\star}_{SPS}$ fouled tank	*F*	p	Adjusted *F*	Adjusted p
2012	0.6	0.7	0.7	0.5	0.38	0.770	0.12	0.944
2013	2.1	0.7	0.5	0.1	22.92	**<0.001**	1.71	0.212

at the location of the enclosure (median = 189 spat m^{-2}) than outside (6 spat m^{-2}; Wilcoxon rank sum test, $X^2 = 7.99$, p = 0.005; Fig. 8). After winter, 61% of the spat remained on the reef, lowering oyster densities to 115 juveniles m^{-2} prior to the adding the 2013 cohort (Fig. 8). Adding the second cohort of larvae in 2013 led to a supplement of 23 spat m^{-2} 1 mo post-set. Although this value only represented 12% of the initial seeding in 2012 (189 m^{-2}), no spat were observed on substrate outside the location of the enclosure, so the addition represented a supplement to the reef that did not occur naturally.

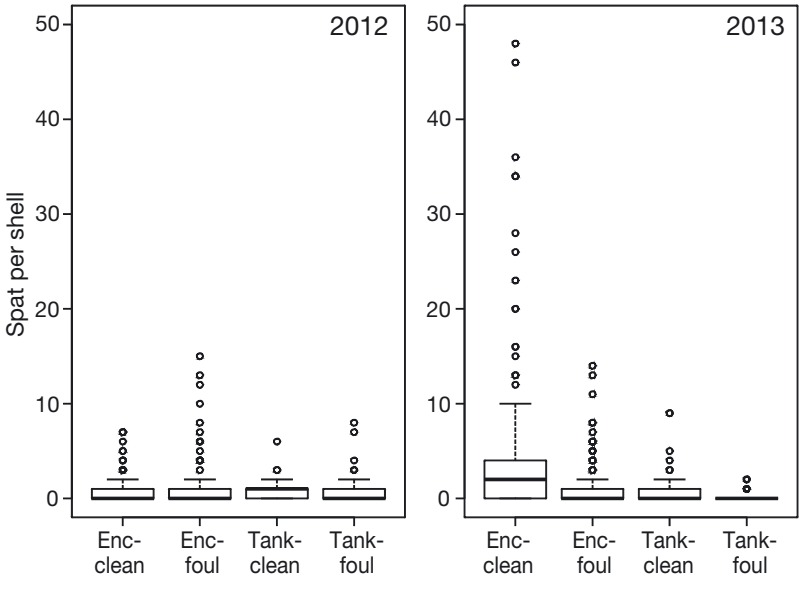

Fig 7. Spat per shell in the off-site raceway at SERC 1 mo post-set. See Fig. 5 legend for explanation of box and whisker plots. No significant difference in SPS occurred among the 4 larval setting treatments after 1 mo in either 2012 (mixed model nested ANOVA, p = 0.944) or 2013 (mixed model nested ANOVA, p = 0.212)

DISCUSSION

Water quality and initial set

In 2012 high salinity in Chesapeake Bay was favorable for *Crassostrea virginica* set, with spatfall intensity throughout the bay roughly 3 times the running median (Southworth & Mann 2012, Tarnowski 2013). Spatfall in the Patuxent River adjacent to St. Leonard Creek, ranged from 8 to 70 spat per bushel (Tarnowski 2013), or 16 to 138 spat m^{-2}. However, in 2012 the reef placement and the experiment occurred relatively late in the typical *C. viriginica* setting season in Chesapeake Bay (Kennedy 1996). The late set, combined with the results that fewer than 0.001 SPS were observed on spat collector bags placed outside the enclosure and that no spat were found on the pier con-

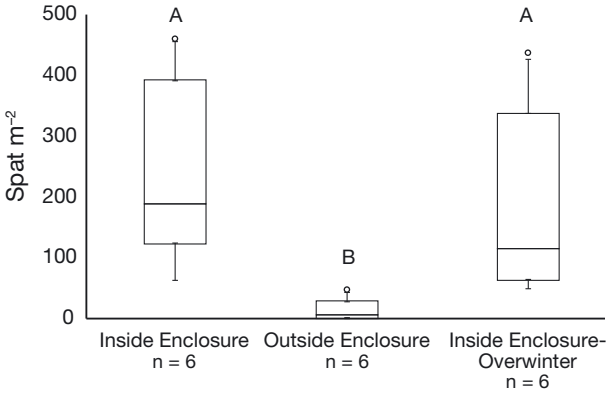

Fig. 8. Spat density of 2012 cohort on the reef 1 mo post-set, inside and outside the enclosure, and in spring 2013. See Fig. 5 legend for explanation of box and whisker plots; letters indicate statistical differences. Spat densities at the location of the enclosure (median = 189 spat m^{-2}) were significantly higher than outside the enclosure (median = 6 spat m^{-2}) (Wilcoxon rank sum test, p = 0.005. Overwinter survival was 61% (median 115 spat m^{-2})

trols, suggest that the spat found on the reef and that remained 1 mo post-set resulted directly from the experimental larval addition, rather than natural recruitment. In 2013, bay-wide set of *C. virginica* was average (Southworth & Mann 2013, Tarnowski 2014), with approximately 5 spat per bushel found in the mid-Patuxent River, or 10 spat m^{-2}. Though in 2013 our larval set experiment took place earlier during the recruitment season and a small spat set occurred in the Patuxent that year, spat were neither found in spat collector bags placed outside the enclosure during the experiment, nor in the MSU-PEARL pier control bags. Natural set in the Patuxent River was less than half the magnitude found at our study site (23 spat m^{-2}), and no recently-set spat were found outside the location of the enclosure 1 mo post-set. This suggested that our direct set of larvae caused the increased spat densities observed at the location of the enclosure in 2013, as it had in 2012.

In both years, larval setting efficiency was higher in the enclosure than in the shoreside tanks used to represent remote setting. It is well recognized that spat setting efficiency and survival can vary considerably among sets (Bohn et al. 1995, Congrove et al. 2009). Our study was limited to 2 larval additions, so it did not illustrate the full range of setting efficiencies possible. The enclosure setting efficiencies of around 15% found in our work, however, fall within the range of published estimates of remote setting success (Bohn et al. 1995, Congrove 2008). Nevertheless higher efficiencies, such as the 20 to 30% suggested by Supan (1991) would prove more cost effective for both restoration and aquaculture. Devakie & Ali (2000) demonstrated that storing *C. iredalei* larvae longer than 48 h throughout a range of temperatures significantly reduces oyster set and post-set survival. Therefore, the 4 d larval storage period may have lowered set rates in our experiment. High variability in SPS also occurred among sample trays, bags, and quadrats within a given shell type condition. While SPS likewise varies among shell valves in traditional remote setting operations, aeration of tanks has been suggested to more evenly distribute spat, lowering conspecific competition, and therefore mortality (Supan 1991).

Setting efficiency in the field enclosure may have been limited by predation on larvae. The 2013 experiment co-occurred with a bloom of the sea nettle *Chrysaora quinquecirrha,* a voracious predator of zooplankton (Breitburg & Fulford 2006). While 13 nettles were removed from the enclosure prior to adding larvae, many more likely remained. In a similar manner, macrofauna present on the shell base may have constrained space for larval settlement on the reef in 2013. While some benthos facilitate set of oyster larvae, high levels of organic matter have been shown to deter settlement via water-borne cues (Campbell et al. 2011). Finally, sediment deposition on the reef itself may have lowered optimal set of additional larvae in 2013. During quadrat sampling on the reef in May 2013, sediment deposition was evident, and this may have reduced the area available for larval attachment later in the summer. If resuspended, the sediment could have also smothered young oysters or reduced their feeding efficiency, lowering survival in the post-set period (Wilber & Clarke 2001, Burke 2010, Whitman & Reidenbach 2012).

One month survival

In both years, quadrat sampling demonstrated that spat densities remained significantly higher in the location of the enclosure than outside it 1 mo post-set and remained high over winter (Fig.8). This suggested that direct setting led to sustained increases in both spat set and juvenile recruitment. Densities also met desired oyster restoration best-practice metrics of over 50 oysters m^{-2} (Baggett et al. 2014), remaining high for at least 8 mo, despite evidence of high spat mortality immediately post-set.

Similar to previous studies in diverse invertebrate taxa (Hunt & Scheibling 1997, Devakie and Ali 2000, Congrove 2008, Edwards et al. 2015), low survival occurred in the first month post-set, but increased

substantially thereafter. Wieland (2007) assumed 60 to 70% survival of oysters between planting (2 to 4 mm shell valve height) and harvest. In our study this roughly equates to the period immediately following initial set through harvest. However, Wieland's estimates come from oysters caged in nurseries to reduce predation, a practice that reduces mortality, but increases materials handling costs. In contrast, Supan (1991) suggests that 70% survival occurs during the nursery period, but only 15 to 25% survival between the nursery period and harvest. Using the overwinter survival rate from the current study (60%) and applying it each year post-set for 3 yr, a survival rate of 22% would occur during a period comparable to Supan's grow-out period.

As with initial larval set, post-set mortality may have been driven by competition for space, or predation (Carroll et al. 2015, Knights et al. 2012, Munroe et al. 2015). In both years, the fouled shell treatments contained valves populated with a 1 mo biofouling community, yet in 2013 the fouled shell treatments and the quadrat samples also contained shell base with communities grown in the creek for a full year. This established benthic community, including the bryozoan *Victorella pavida,* ascidians (*Cliona intestinalis*), and barnacles (*Balanus* spp.) have been shown to dominate space on benthic substrata (Sutherland & Karlson 1977, Osman et al. 1989). Other benthic fauna, such as mud crabs (*Rhithropanopeus harrisii*, *Eurypanopeus depressus*, *Dyspanopeus sayi*, *Panopeus herbstii*), blue crabs *Callinectes sapidus*, polychaete worms (*Polydora cornuta*) and flatworms (*Stylochus* sp.) all prey upon oyster spat, and therefore lower recruitment (Supan 1991, Newell et al. 2000, 2007, Rodney & Paynter 2006, Congrove 2008, Barnes et al. 2010, Campbell et al. 2011, George et al. 2014). As many of these taxa were observed in this study, they likely caused spat mortality.

Survival of spat transported to the off-site grow-out raceway varied greatly, yet was comparable to, or slightly lower than that observed on-site. As newly-set spat are extremely fragile (Jones & Jones 1988), and spat in our study were transported soon after set, these values likely underestimated survival during typical transport to nursery sites. Additionally flow rate, and therefore food supply, likely differed between the laboratory raceway at SERC and in St. Leonard Creek (Grizzle et al. 2008), and it is possible that reduced seston availability lowered oyster survival off-site.

In 2013, we supplemented the reef emplaced in 2012 with a second cohort of approximately 20 additional spat m^{-2}. Although the supplement represented only 12% of the spat densities produced the previous year, the method did not require additional substrata to be placed in the creek, or any hatchery-produced seed to be transported to the study site. As changes in the carbonate chemistry and shell budget of eutrophic estuaries are reducing the clean oyster shell cultch available for traditional remote setting practices (Powell et al. 2006, Powell & Klinck 2007, Waldbusser et al. 2011, 2015), setting spat on alternative substrates has become more common (Nestlerode et al. 2007, Black 2011, Theuerkauf et al. 2015). While handling and transport of some materials, such as clamshell, may be comparable to oyster shell base, other substrata used for setting larvae, such as cobble, reef balls, and Oyster Castles® (Allied Concrete) could incur higher costs. Direct setting of larvae on alternate substrata in the field may reduce transport costs associated with moving cultch to setting tanks and then transporting the seed to the field sites in areas of low natural recruitment. In the case of bottom-harvest aquaculture sites, which may require only a single cohort to be set in a given year, it may prove tractable to set a small portion of a lease at a time, and repeating the process over the course of a few weeks. However, timing of this procedure should take into account not only favorable water quality conditions (temperature, salinity, dissolved oxygen), but also the predator suite occurring at the site.

In conclusion we have shown that adding larvae directly to an emplaced reef or aquaculture site could provide spat sets comparable to traditional remote tank setting techniques. While previous studies have demonstrated success setting bivalve larvae on substrata in shallow mesocosms (Arnold 2008, Burke 2010, Theuerkauf et al. 2015), our work is the first to quantify direct sets of *Crassostrea virginica* larvae on a 65 m^2 reef and in subtidal waters deeper than 2 m, scales more applicable to restoration and aquaculture. The goal of many restoration initiatives in Chesapeake Bay is to produce reefs with over 50 oysters m^{-2} (Baggett et al. 2014), and those densities were reached incorporating direct larval set in this study. More importantly, the densities were maintained for a full year while negating the need to add additional substrata. Short-term deployment of the larval enclosure precluded the need to aerate the system, even during a time period in which hypoxic conditions are typically found, yet a method to more evenly distribute the larvae over the enclosure may be needed to reduce overcrowding on shell valves and potential intraspecific competition post-set. However, by designing and engineering an enclo-

sure and mooring system capable of withstanding moderate tidal, wave, and ship-wake activity typical of coastal tributaries (Fredriksson et al. 2016), direct setting may be applied on a small scale to tributaries with similar features, and potentially be scaled up to accommodate larger restoration sites or aquaculture bottom leases.

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