Fragment reattachment, reproductive status, and health indicators of the invasive colonial tunicate *Didemnum vexillum* with implications for dispersal

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Abstract The invasive colonial tunicate *Didemnum vexillum* is now widespread in coastal and offshore waters of New England, USA. *D. vexillum* can inflict ecological and economic damage through biofouling and habitat modification. Natural and anthropogenic processes that fragment colonies of *D. vexillum* may be accelerating the spread of this invader. Reattachment success and fragment viability were confirmed in the laboratory after four weeks of suspension in experimental aquaria. The shape of suspended *D. vexillum* fragments progressed from flattened to globular spheres and then flattened again after reattachment to the substrate. Reproductive activity, confirmed by the presence of eggs and larvae, was observed for fragments suspended up to three weeks suggesting that *D. vexillum* is capable of reproducing while in a fragmented, suspended state. An index of colony health was used to monitor change in *D. vexillum* health while in suspension. Overall, colony health declined with time in suspension although colonies that appeared dead (black and gray in overall color) still contained a substantial number of healthy live zooids. These results suggest that activities that cause fragmentation can significantly facilitate the spread of *D. vexillum*. Coastal managers should consider reducing or eliminating, when practical, activities that return fragmented colonies of *D. vexillum* to the water. In-water cleaning of biofouling and dredging are likely expediting the spread of this invasive species unless biofouling can be contained and removed from the water.

Keywords: *Didemnum vexillum*, Invasive species, Tunicate

Introduction

Tunicates can be nuisance invaders owing to their fouling of both natural and artificial surfaces and overgrowth of sessile invertebrates (Whitlatch and Osman 2000; Pederson et al. 2005; Bullard et al. 2007a; Lambert 2007; Dijkstra et al. 2007; Valentine et al. 2007a; Valentine et al. 2007b). The potential ecological impacts of invasive tunicates include alterations to biodiversity and ecosystem function (Stachowicz et al. 2002; Dijkstra et al. 2007; Osman and Whitlach 2007). Of particular economic concern are tunicate species that foul aquaculture and commercial fishing gear (Locke et al. 2007; Carman et al. 2010). Economic losses can be substantial for some commercial fishing sectors, such as the aquaculture industry, where heavy biofouling can reduce shellfish growth rates and increase mortality (Coutts and Sinner 2003; Guenther et al. 2006; Adams et al. 2011).

One of the more notorious tunicate invaders, the colonial tunicate *Didemnum vexillum* Kott, 2002, is apparently native to the northwest Pacific Ocean (Stefaniak et al., 2009; Lambert, 2009). *D. vexillum* has been introduced to coastal and offshore waters of New Zealand, both east and west coasts of the U.S., and the west coast of Canada, and Europe (Coutts and Sinner 2003; Bullard et al. 2007a; Stefaniak et al. 2009). This species reaches high densities and inhabits a wide variety of habitats including cobble rocky bottom and other hard substrates. It can live as an epibiont and is common on hard, man-made surfaces. Ecological effects of *D. vexillum* include an increase in benthic polychaetes (Lengyel et al. 2009, Mercer et al. 2009) and the potential decrease

64 in densities of bivalves, specifically the commercially important bay scallop (*Argopecten*
65 *irradians irradians*) and sea scallop (*Placopecten magellanicus*) (Morris et al. 2009).
66 Like all colonial tunicates, *D. vexillum* reproduces both sexually and asexually, by
67 budding. Valentine et al. (2009) documented that *D. vexillum* release larvae in New
68 England waters for 3.5 to 5 months at temperatures from 14 to 20ºC. While dispersal to
69 new habitats can occur via larval releases from adult colonies, didemnid colonies can
70 also invade by anthropogenic vectors (e.g., sea chests, boat hulls) and natural or human-
71 induced fragmentation (Ryland and Warner 1986; Stoner 1989; Lengyel et al. 2009).
72 Fragmentation (the breaking apart of tunicate colonies into smaller fragments) of
73 didemnids can occur during many human activities including dredging, cleaning boat
74 hulls and floating docks, and defouling of commercial fishing and aquaculture gear.
75 The influence of temperature and suspension duration on reattachment of
76 didemnid fragments in New England is not well understood. Earlier reports have
77 documented fragment reattachment success in summer months in Connecticut
78 (McCarthy et al. 2007; Bullard et al. 2007b) and Massachusetts (Valentine et al. 2007a);
79 however, no prior studies have documented reattachment, reproductive status, or
80 general fragment colony health during fall temperatures. Here we report fragment
81 reattachment success, reproductive activity, and a general colony health assessment for
82 *D. vexillum* fragments suspended in the water column during fall ambient water
83 temperatures at Woods Hole, Massachusetts.

Methods

We created fragments of *D. vexillum* and suspending those fragments in the water column using aquaria with flow-through water and aeration. Fragments were held in suspension from one to four weeks after which they were placed in the field in individual containers and monitored to determine reattachment success. Reattachment success for each suspension duration, changes in fragment morphology, and health and reproductive status of fragments during this experiment were recorded.

Fragmentation of *D. vexillum*

Colonies of *D. vexillum* were cultured on 12 cm^2 PVC plastic gray settling plates and suspended at 3 m depth from a dock located at Woods Hole Oceanographic Institution (WHOI), Woods Hole, Massachusetts from July to September 2008. On September 23, eight settling plates were removed and transported to the laboratory equipped with flowing seawater. Colony fragments were removed from the settling plates using a stainless steel spatula and unwanted organisms (other tunicate species, barnacles, anemones, and other macro-invertebrates that attached to the settling plates) were removed as completely as possible without damaging the colony. Any remaining biota on the colony were noted.

Equal-sized fragments (1 cm^2) were then cut from the colonies with a razor blade and divided among three flow-through 38-L glass aquaria at a density of 90 ± 1 fragments per aquarium. Each aquarium was supplied with a flow-through seawater

rate of 9.1-L per min. Four aeration stones were activated in each aquarium to ensure adequate aeration and mixing. The water flow created currents that kept the fragments suspended in the water column. Aquaria were monitored regularly. Seawater flow was continuous throughout the study except during a five-hour interruption at the mid-point of the experiment owing to maintenance of the seawater intake system. Aeration was continued during this period. We assume no impact from this brief interruption as the fragments settled on the bottom, but did not attach.

Husbandry and Water Quality

Seawater temperature was maintained at ambient levels by maintaining a high turnover rate in the aquaria of 15 water changes per hour using a flow-through supply of seawater. The mean ± SE water temperature in the aquaria for the entire suspension period was 17.7 ± 0.46 °C. The maximum observed temperature was 19.1 °C (Sept. 30) and the minimum temperature was 16.1 °C (Oct 21). The seawater intake for the lab and at the dock where the fragments were placed is in the same general area so that the fragments were exposed to the same nutrient, salinity (31-32 psu), and temperature regimes.

Fragment reattachment experiment

A field experiment was used to determine fragment reattachment success. Fragments (n = 10 per aquaria) were removed each week for four consecutive weeks, thus providing fragments that had been suspended for one, two, three, and four weeks (WS).

Fragments were removed from each of the three aquaria and placed individually into separate round plastic containers (dimensions = 5.5 cm height, 7 cm bottom diameter, and 9 cm top diameter) with ten perforations for water flow. An equal number of control fragments (total n = 120) were excised on the day of sampling from nearby natural colonies attached to hard (rock or rope) substrate and similarly placed in plastic containers. Experimental and control containers were affixed in alternating positions along a line that was then suspended below the water surface at a depth of approximately 3-4 m. The controls used in week one were scraped from rock; all others were taken from floating docks. All experimental and control fragments were given one week to attach (or not) to the inside of the container.

After one week, the containers were opened and fragment reattachment success was determined as attached, weakly attached, or not attached using a gentle flow of seawater from pipettes. First, a 10 ml plastic pipette (inner tube diameter ca. 14 mm, tip diameter ca. 2 mm; Bullard et al. 2007b) was used. If the attachment was not disrupted by water flowing from the plastic pipette, a standard 10 cm (inner tube diameter ca. 6 mm, tip diameter ca. 2 mm) glass pipette with rubber bulb was used. The test of reattachment was done by placing the pipettes in the container, nearly touching the colonies so that a flow of seawater was applied while the colonies were submerged. Weakly attached was recorded if the colony remained attached when tested with the plastic pipette but became loose when tested with the glass pipette. A Student's t-test was used to compare attachment level (attached, not attached, weakly

attached) between each treatment for each week with a $p < 0.05$ considered statistically significant.

Fragment health assessments

Fragment health of living colonies was assessed visually by the naked eye and with a dissecting microscope. Fragment subsamples comprised a range of sample sizes across the four sampling times (n = 1-3 fragments per experimental aquaria and n=3 fragments from the controls) that were assessed for any changes in fragment health. Fragment overall health was assessed with the following indicators: 1) integrity of zooids (whether necrosis of tissues was observed), 2) changes in color and texture of the colony's exterior, 3) presence/abundance of detritivores and carnivores indicative of unhealthy colonies, and 4) buildup of detritus including fecal pellets and dead tissue within the colony (see Table 1 for complete list). A health index was developed based on health ratings of good, fair, poor where fragments exhibiting viable zooids, little evidence of detritivores, and little detritus were given a health rating of good, fragments with any two negative indicators were given a rating of fair, and fragments with more than two negative indicators were given a rating of poor. Observations of fragment shape and size were recorded as the samples were placed in the plastic containers and again after a week when they were tested for reattachment in the containers.

To assess reproductive status, the same subsamples were dissected under a microscope and the presence of eggs or larvae was recorded. Percent number of fragments containing eggs or larvae was determined for both experimental (mean n =

7.5 ± 1.3 standard error fragments per week) and control (mean n = 5 ± 1.2 standard error fragments per week) treatments at the end of the reattachment period.

Results

Fragment reattachment

Fragments of *D. vexillum* demonstrated the capacity to reattach after being suspended for four weeks. Overall attachment, including both attached and weakly attached, was highest for the 1 week (WS) group, with 62% of the fragments exhibiting attachment and 38% not attached. Attachment declined to 36% for the 2WS, 27% for 3WS, and 33% for the 4WS groups (Fig. 1). Overall attachment success of control fragments was lower for the first week (attached + weakly attached = 23%), but increased to 73, 57, and 50% for the remaining weeks. Weekly differences in attachment were detected between the suspended and control fragments. A higher number of control fragments were attached at week 2 ($p = 0.01$) and week 4 ($p = 0.001$) when compared to the suspended fragments, however, a higher number of suspended fragments were attached during week 1 ($p = 0.04$) and week 2 ($p = 0.02$).

Some of the sample containers with colonies were held in a water table in the laboratory for as long as 24 hours after removal from the field, pending dissection for observations of health and reproductive condition. During this holding period, we observed that at least four fragments that had originally attached to the bottom of the holding container were now attached instead to the lid of the container. This

observation suggests that colonies can be dislodged from a substratum and still reattach in a new location within a microhabitat within 24 hours and without changing shape.

Fragment health and reproduction

Attached fragments displayed asexual growth, whereas unattached fragments exhibited no observable new colony growth. In general, the overall health of the fragments declined over time. Some colonies that were covered with detritus and were black in color appeared dead during the gross examination; however, they contained substantial numbers of healthy zooids when viewed under the microscope. The reproductive assessment indicated that fragments in suspension and reattached could remain reproductively active for four weeks. The percent of reproductively active fragments decreased over time from 80% during week 1 to 25% after week 4 (Fig. 2).

Detritivores and carnivores including nematodes, flatworms, harpacticoid copepods, ciliated protozoans, and entoprocts (*Barentsia* sp.) were observed on both the experimental and control fragments.

Fragment morphology

Nearly all of the experimental fragments (1WS-4WS groups) changed in shape after being fragmented and placed in suspension (Fig. 3). The general shape progression was from flat (at the time of fragmentation) to enrolled to round or globule while in suspension and then flat again once the colony fragment had reattached. Some of the fragments ($n > 10$) fused together while in suspension in the aquaria.

215 Shape change was due to fragments becoming enrolled (Fig. 3), with damaged
216 tissue (original attachment surface) facing inward and therefore isolated from the
217 remaining tissue and not interacting with potential substrata. Many of the samples
218 showed complete closure of the globule, while others were open on one or both ends.
219 Following one week of suspension, about 80% of the fragments enrolled into globules,
220 whereas 100% were globules for the 2WS, 3WS, and 4WS groups. When attachment
221 occurred, the globulized fragments flattened and attachment occurred at new growth
222 regions. The greatest observed change in fragment surface area was quintuple in one
223 week.
224 None of the control fragments enrolled into globules during their week-long
225 deployment in containers. Only slight rounding at the colony margins was observed.
226
227 **Discussion**
228 *D. vexillum* is continuing to spread causing a wide-array of economic and
229 ecological impacts (Carman and Grunden 2010; Adams et al. 2011; Cohen et al. 2011;
230 Dijkstra and Nolan 2011). This study provides insight into a mechanism of dispersal that
231 can increase the success of *D. vexillum* as an invasive species. After four weeks in
232 suspension followed by a week of opportunity to reattach to a substratum, asexual
233 growth and production of larvae within the new tissues of a colony were observed.
234 Thus, *D. vexillum* can complete a generation cycle within seven days or less even at fall
235 water temperatures in New England. A small number of zooids, perhaps one, can form
236 a new attachment and grow into a colony; therefore, very small fragments as well as

237 suspended fragments can be propagules for dispersal. Fragments can form new shapes
238 thus demonstrating remarkable plasticity. The ability of *D. vexillum* fragments to repair
239 themselves by walling off damaged parts while free-living in suspension, and then
240 reattaching, increases their successful establishment in new habitats.

241 Organisms such as colonial tunicates with multiple reproductive strategies that
242 can self-fertilize are generally good colonists (Sakai et al. 2001; Bock et al. 2011). While
243 natural dispersal is not considered a major contributor to ascidian spread, it can
244 increase local abundance (Bock et al. 2011). The natural generation and spread of
245 colony fragments have often been cited as a potentially important mechanism of
246 secondary spread (Lambert 2005; Carver et al. 2006). The observations herein provide
247 the first long term assessment of fragmentation and reattachment for *D vexillum*. While
248 interpretation of the results of this study is limited to laboratory based observations, the
249 implications and ramifications of these observations are large. Future efforts to better
250 understand fragmentation and the role it may play in the life history and spread of *D.*
251 *vexillum* are needed. It should be noted that the first week controls were from a
252 different substratum (hard rock) than the other controls. The scraping of the samples
253 from the rocks may have disrupted the ventral surface of the colonies, hence may have
254 damaged them such that their attachment rate was reduced.

255 The observed changes in fragment morphology during this study demonstrate
256 the ability of *D. vexillum* to re-shape itself based on substrate type or the lack thereof.
257 These characteristics of *D. vexillum* have not been shown before. An enrolled didemnid
258 fragment may be an adaptation for dispersal or self-protection while a flat fragment

may be more adapted for attachment. The overall decline in fragment health was not surprising given the length of time in suspension. The health indicators reported here can serve as a guide for future assessments of dideminid colonies across various environmental regimes and laboratory experiments.

The cleaning of marine aquaculture gear, lobster pots, and boat hulls has the potential to cause local multiplication of colonies and to exacerbate the damage they cause. It is important that cleaning of fishery or aquaculture gear take place on land whenever possible and that the fouling removed be dried or disposed of on land. Hopkins et al. (2010) recommended that if defouling gear in-water is necessary, the reproductive state, season, and underlying seabed type should be considered. Our results agree with Hopkins et al. (2010) but expand the period of potential reattachment well into the fall in New England. Further research is needed on the relative contribution of *D. vexillum* fragmentation to increases in local abundance, the apparent ability of *D. vexillum* to reproduce while in suspension, shape plasticity, the reattachment rate of fragments, and general colony health throughout the entire year.

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385 Table 1. Positive (+) and negative (-) health indicators observed for *D. vexillum* while in

386 suspension and during reattachment trials.

387

388

Health indicator	Type
Tunic	
Clean exterior	+
Organic buildup (fungae, algae, bacteria)	-
Dedifferentiation of zooids	-
New growth at edge of colony	+
Brown and without spicules	-
Tunic peeling	-
Attached diatoms	?
Dark inclusion bodies	-
Blackened surface	-
High proportion of tightly packed spicules	+
Interior	
Contains debris	-
Buildup of fecal pellets	-
Brown/dedifferentiated zooids	-
Zooid structure is largely transparent	-
Free-living organisms associated with the colony	
Harpacticoid copepods	-
Flatworms	-
Ciliated protozoans	-
Nematodes	-

389

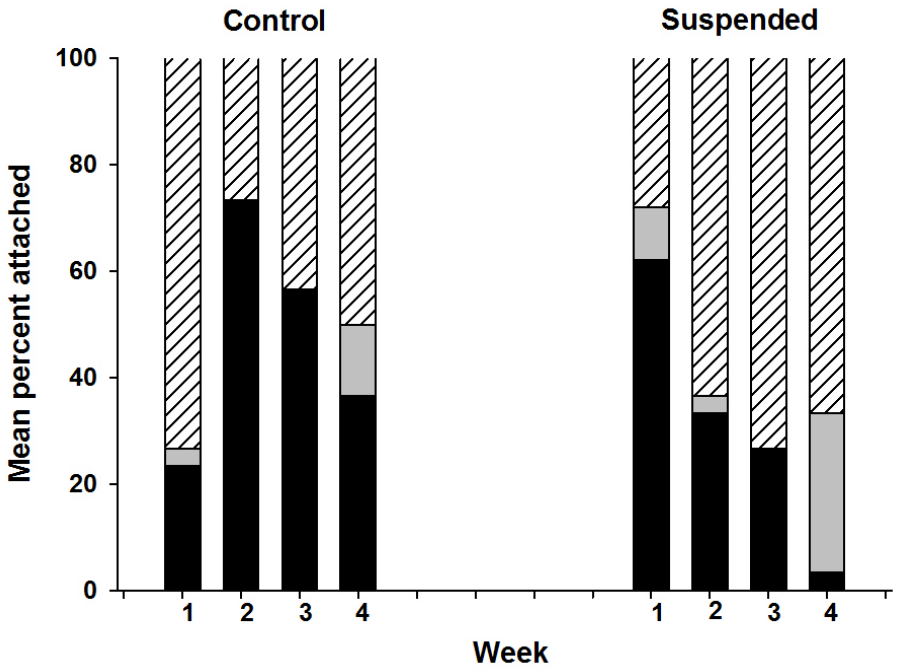

Fig. 1. Mean percent of attached (black bars), weakly attached (dark gray bars), and not attached (lined bars) fragments for each week in the control and suspended treatments.

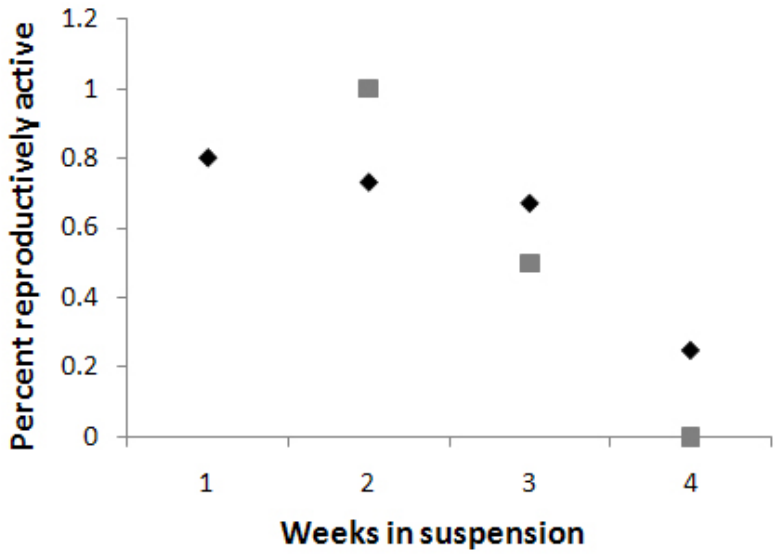

Fig. 2. Percent of reproductively active formerly suspended (diamonds) and control (squares) *D. vexillum* fragments as defined by the presence of larvae or eggs. Note that eggs and larvae were most often found in new growth regions.

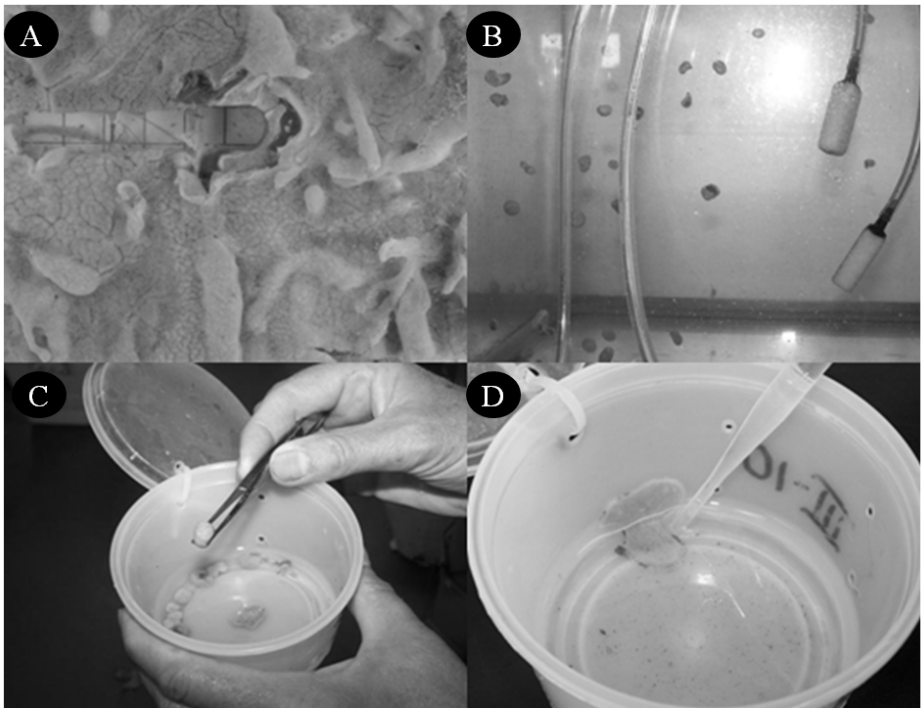

Fig 3. Photographs demonstrating differences in shape morphology of *D. vexillum*. A) flattened morphology prior to fragmentation, B) globule morphology while in suspension, C) globule morphology when placed into holding container, and D) flat morphology including new growth upon reattachment.