

## Adventures of a sea squirt sleuth: unraveling the identity of *Didemnum vexillum*, a global ascidian invader

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### Abstract

The magnitude of the worldwide invasions of *Didemnum vexillum* Kott, 2002 has taken a number of years to be comprehended. During the past 15 years, it has been identified as different species depending on its location—*D. carnulentum* on the U.S. west coast, *D. lutarium* or *D. vestum* in New England, *D. lahillei* or *D. helgolandicum* in France and the Netherlands, *D. vexillum* in New Zealand, *D. pardum* or *D. moseleyi* in Japan. A number of recent publications refer to it as *Didemnum* sp. or *Didemnum* sp. A. This paper presents a chronology of the steps in the development of our awareness and understanding of this species based on comparative morphology and genetics, and lists invaded regions and the approximate minimum length of time it has been known in each area. Evidence is presented that *D. vexillum* may have originated in Japan. The importation of vast quantities of Japanese oysters and spat into many countries prior to the 1960s is discussed but eliminated as a likely vector because there are no reports of a sudden didemnid ascidian appearance prior to the 1970s. Introductions (including to the type locality in New Zealand) are very likely due to shipping (either via hull or sea chest fouling), with subsequent local spreading by fouled recreational craft, barges, etc., drifting and reattachment of dislodged fragments, and movements of fouled aquaculture stock and gear. Based on morphological and genetic comparisons of hundreds of world-wide samples, museum type specimens, and anecdotal information on the presence of this species in various locations over several or many decades, the valid name is concluded to be *Didemnum vexillum* Kott, 2002 due to the lack of any pre-existing published description. *D. vestum* Kott, 2004 is synonymized under *D. vexillum*.

*Key words:* *Didemnum*, fouling, marina, tunicate, ascidian, invasions, nonindigenous, *col*

### Introduction

"If you do not know the names of things, the knowledge of them is lost, too." (Linnaeus, 1751).

Fundamental to understanding the scale and impacts of biological invasions in the sea is a clear and accurate resolution of the identification of invasive species. There is perhaps no better example of this need than the saga of the global spread of a species of ascidian (sea squirt) in the genus *Didemnum*, whose appearance in both inshore and offshore areas around the world

commencing in the 1970s and 1980s has been greeted with labyrinthian and obfuscated taxonomy. My goal here is to attempt to sort out and clear up the taxonomy, re-tracing a complex biogeographic and systematic road.

In August 2000 during a rapid assessment survey for invasive marine species in Massachusetts, large abundant colonies of a *Didemnum* sp. were observed at several sites. I tentatively identified the specimens as *D. lutarium*, a species described from New England by Van Name in 1910. The following year a very similar species was observed for the first time in New

Zealand fouling boat hulls and harbor structures in Tauranga (May 2001) and Whangamata Harbours (Sept. 2001) (B. Coffey pers. comm.). It was subsequently described as a new species, *Didemnum vexillum*, by Kott (2002), who declared it native to New Zealand rather than an introduction. A sample of the New England *Didemnum* was sent to New Zealand for comparison, and forwarded to Kott, who described it (Kott 2004) as an overlooked new species native to the northeast U.S., *Didemnum vestum*. Meanwhile, I observed similar-appearing colonies in Brittany in July 2002 (unpubl. obs.) that were tentatively identified by F. Monniot as *D. lahillei* Hartmeyer, 1909, a native French species. Further complicating the story, recently appearing colonies in other parts of northern Europe were being referred to also as *D. lahillei*, or as *D. helgolandicum* Michaelsen, 1921. In May of 2003 I observed numerous large lobed colonies fouling floating docks in San Francisco, Bodega and Tomales Bays in California, and divers reported it spreading rapidly on the subtidal portions of a breakwater at the entrance to Bodega Bay, where it had only recently appeared. Various similar specimens from California collections of the previous few years, initially identified by me as the California species *D. carnulentum* based on resemblances to the published description by Ritter and Forsyth (1917), were upon reexamination found to be the same as all the new samples. T. Nishikawa provided a sample from Ise Bay, Japan, that also appeared to match, which he identified as the native Japanese species *D. pardum* Tokioka, 1962 but which differed somewhat from that species in several morphological characters. With all the uncertainty surrounding the identity of this species, many researchers including myself decided to refer to it as simply *Didemnum* sp. A or *Didemnum* sp. (see the numerous papers in Journal of Experimental Marine Biology and Ecology 342 (1)).

Surveys were undertaken to establish the distribution on both coasts of the U.S.; see Bullard et al. (2007a) for complete listing of U.S. and British Columbia records known at the time of publication. (The earliest Puget Sound listing has now been moved back from 2004 to 1998 as noted below.) Most significantly, over 230 km<sup>2</sup> of the Georges Bank is now covered 50-90% by this species (Valentine et al. 2007a). With an increase in awareness and publicity, new records including Ireland (Minchin and Sides 2006) are being added as this species continues

to expand its worldwide distribution (US Geological Survey 2008). With the realization that all these populations were morphologically similar and thus possibly all the same species, DNA sequencing was vital to test this hypothesis.

V. Webb, a molecular biologist at the University of Auckland, New Zealand, was the first to sequence the 18S rDNA from New Hampshire, New Zealand, and Japan samples in 2005. She found 98% sequence identity among them and concluded that they were all the same species (unpublished data). L. Zeng also carried out 18S rDNA analyses using a much larger number of *Didemnum* sp. A samples from around the world, and all were remarkably similar, leading to the same conclusion of conspecificity. However, there is some controversy that 18S rDNA is too conserved to be suitable for making species-level distinctions, though it is useful for constructing phylogenies of higher groupings (Swalla et al. 2000). Between 2003 and 2006 several workers attempted unsuccessfully to sequence the mitochondrial cytochrome oxidase I (*coI*) gene, now generally accepted as a valuable tool for analyzing conspecificity among disjunct populations and also useful for determining the source of new invasions. L. Stefaniak (2009, this issue) decided to utilize the nuclear gene *Tho2*, and this has proven to be diagnostic at the species level. She also succeeded in sequencing the *coI* gene, and has shown that all the populations of *Didemnum* sp. A as well as *D. vexillum* and *D. vestum*, belong to the same species while other known *Didemnum* species tested as outgroups were easily separated. The final tasks, therefore, were to determine the valid species name and the region of origin, now concluded to be *Didemnum vexillum*, most likely originating from Japan, based on the data presented below.

## Methods

Hundreds of samples of *Didemnum* sp. A and other *Didemnum* species were collected over the past 15 years by myself and many other people from numerous locations worldwide (see Acknowledgements): northeast and west coasts of the U.S. including many New England harbors, the Georges Bank, British Columbia, Ireland, France, Netherlands, Japan and New Zealand (see Discussion for more precise locations). Most specimens for morphological analysis were relaxed with menthol crystals in

sea water, fixed and stored in 10% seawater formalin buffered with sodium borate, and examined in seawater, with subsamples fixed directly in 95% ethanol. Some specimens were available only from fixation in 95% ethanol or 70% isopropyl alcohol. Photographs taken by myself were taken with a Nikon Coolpix 4500, if necessary on a Wild M7 S dissecting microscope. Samples for DNA sequencing were fixed directly into 95% ethanol.

Spicules were prepared for SEM as follows: a small piece of fixed tissue was rinsed briefly in distilled water, blotted dry, then burned for ~5 min in a heatproof ceramic dish over a Bunsen burner. Bleach (5% sodium hypochlorite) was added while the dish was still warm, then removed by pipette after 5 min. The spicules were washed several times in distilled water to remove bleach and debris, then dehydrated through a series of 70%, 95%, and 100% ethanol. Spicules were placed on dry SEM stubs in a drop of 100% ethanol which was allowed to evaporate. The stubs were then gold sputter coated on a Cressington 180 with rotary planetary stage and examined in a Zeiss EVO40 XVP SEM at the Santa Barbara Museum of Natural History.

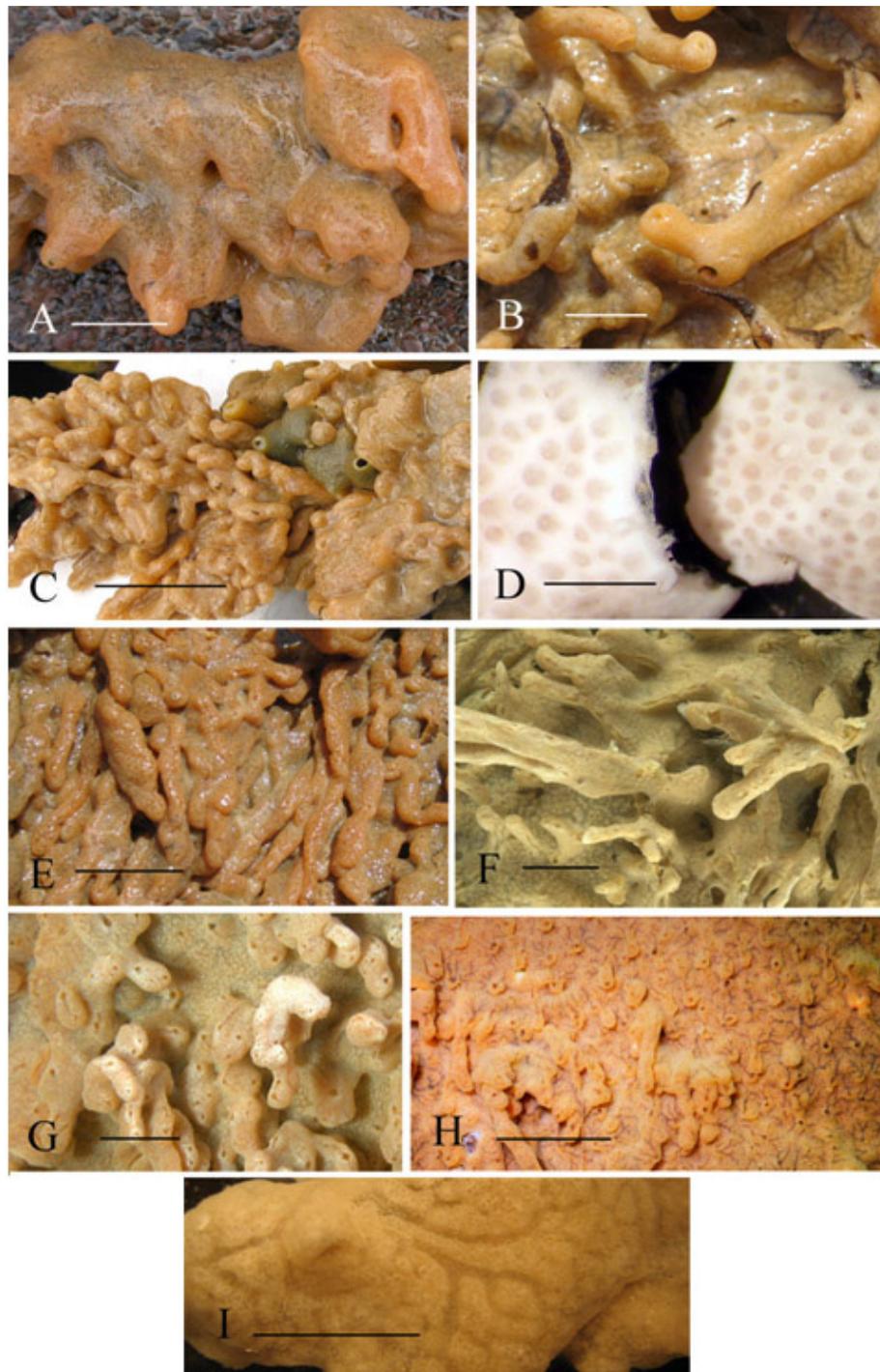
The type specimen and a cotype of *Didemnum helgolandicum* were borrowed from the Zoological Museum of the University of Hamburg; a second cotype (determined to be a piece of the type colony) was borrowed from the Zoological Museum, University of Copenhagen. Preserved specimens of *Didemnum lutarium* from the northeastern U.S., *D. carnulentum* from California and *D. misakiense* from Japan were borrowed from the Smithsonian Institution, National Museum of Natural History. The single colony of *D. misakiense* had been dredged at 62 m depth by the R/V Albatross from Suruga Bay, Shizuoka prefecture, Honshu Island 16 May 1900. *Didemnum candidum lutarium* and *D. albidum* were borrowed from the Huntsman Marine Science Centre ARC, St. Andrews, NB, Canada. Three small pieces of the holotype of *D. vestum* were borrowed from the Queensland Museum, Australia. T. Nishikawa kindly donated samples of *Didemnum* sp. (which he had identified as *D. pardum*) that he collected from low intertidal rocks on Saku Island, Ise Bay, Japan on 31 July 2003. The type specimens of *D. pacificum*, *D. pardum* and *D. areolatum*, all collected in Sagami Bay, Japan, were borrowed from the Showa Memorial Institute, National Museum of Nature and Science, Tsukuba City, Japan. A *Didemnum* sp. specimen #349 was borrowed

from the Oka collection, Dept. of Zoology, National Science Museum, Tokyo. It was collected by Hozawa and Takatsuki on Aug. 2, 1926 off Tsubakiyama, northern end of Natsudomari Peninsula, Mutsu Bay, on *Zostera* sp. "or other materials" (see Nishikawa 1990, p. 103) and preserved in seawater formalin. H. Imabayashi loaned several colonies of *Didemnum* sp. A (that had been identified as *D. moseleyi*), collected from cultured oysters in Hiroshima Bay 27 March 2006. Fresh specimens of Spanish Mediterranean and Atlantic *D. lahillei* were collected by X. Turon and E. Vázquez in November 2004 for comparison with museum specimens of this and other *Didemnum* spp. at the Museum National d'Histoire Naturelle, Paris by F. Monniot and X. Turon.

## Results

Figure 1A-C and E-I and Figure 2 are a comparison of tunic surface patterns of *Didemnum* sp. A samples from a number of locations. The tunic pattern varies somewhat depending on the substrate: in quiet waters hanging off floating docks, large colonies develop long lobes (Figure 1A-C, E, Figure 2B). Small colonies and those growing on flat horizontal surfaces tend to be encrusting (Figure 1F, Figure 2A, C-F). In Figure 1F the colony, from 45 m on Georges Bank, is actually overgrowing a scallop and other organisms encrusting the scallop (see US Geological Survey (2008) website <http://woodshole.er.usgs.gov/project-pages/stellwagen/didemnum/index.htm> for additional photos). Colonies growing on vertical surfaces such as rock walls or cement breakwaters are often encrusting with numerous short lobes with a cloacal opening at the apex of each lobe (Figure 1G, H). Figure 1D is of two small co-type colonies of *D. pardum*; note the dense spicules, the depression over each oral siphon, the absence of aggregations of zooids into groups and lack of meandering dark spicule-free lines present in all the *Didemnum* sp. A photos in Figures 1 and 2 that indicate the cloacal lacuna system and agree closely with the description and illustration for *D. vexillum* (Kott 2002).

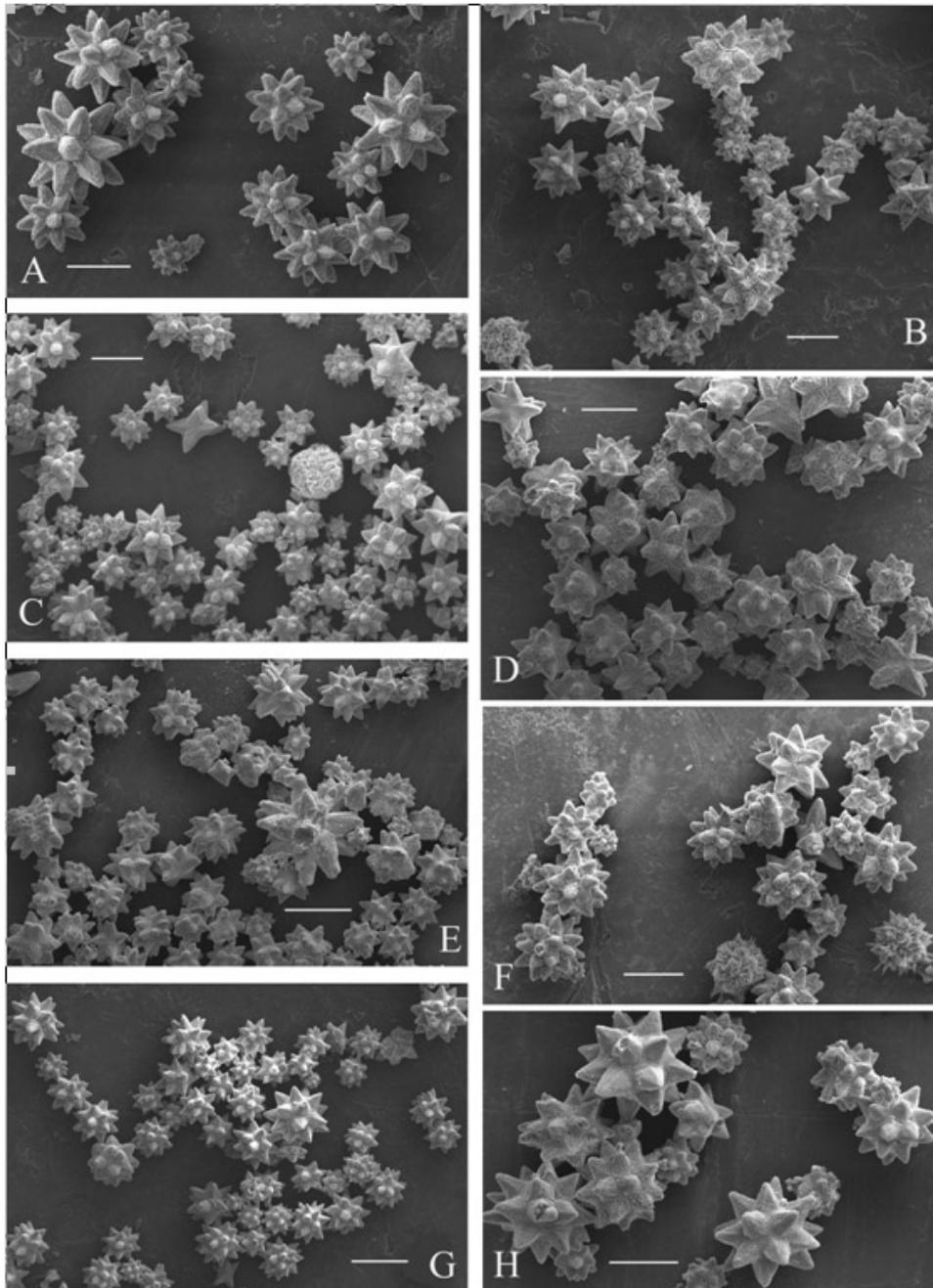
Figure 3 and Figure 4A-F and H compare SEM photos of spicules isolated from a number of *Didemnum* sp. A samples collected worldwide. The spicules in each photo are from a single colony, and show some of the intra-colony



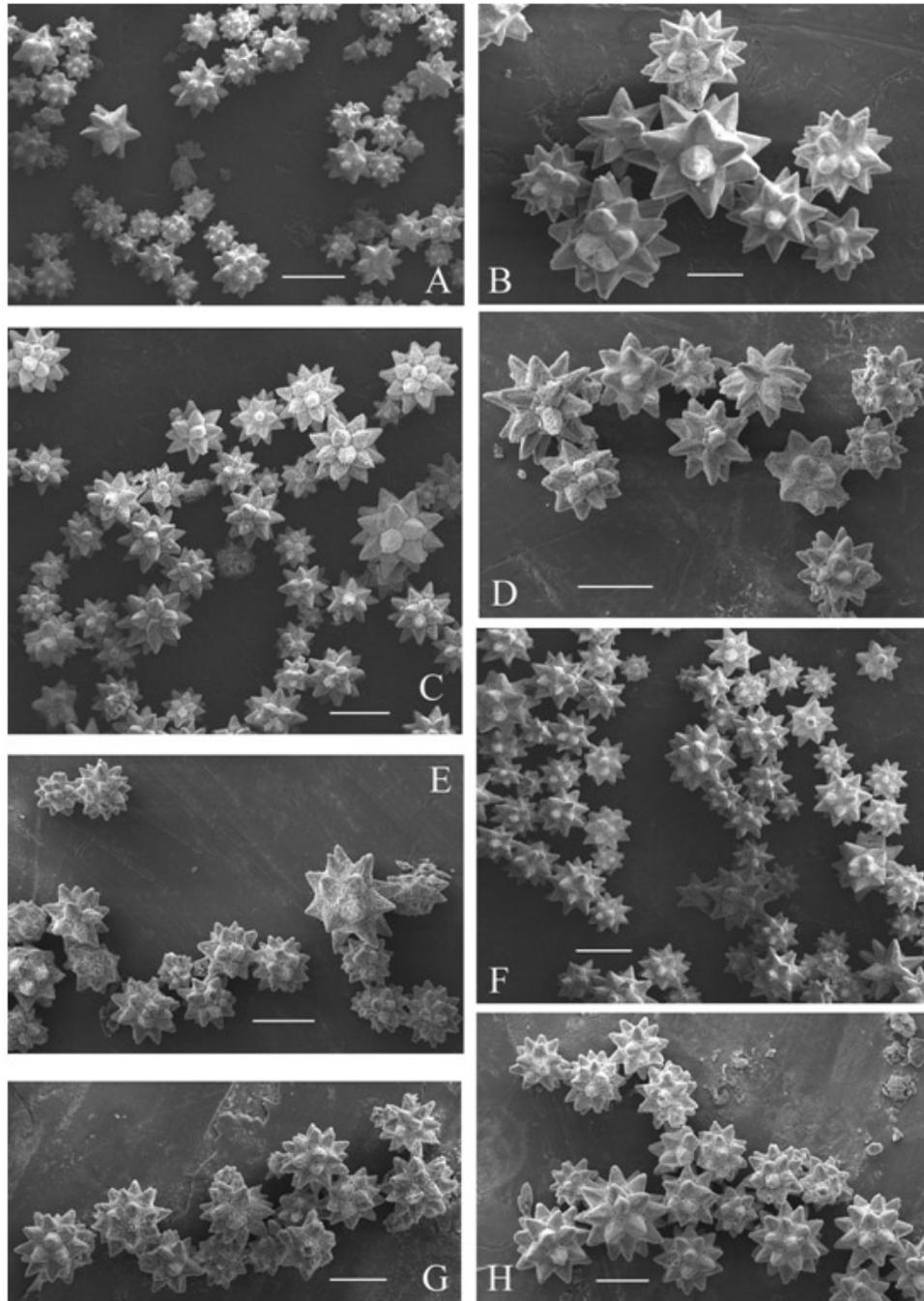
**Figure 1.** A-C, E-H: Tunic surface pattern of *Didemnum* sp. colonies. A) Des Moines Marina submerged line, WA, 2 Aug. 2006; B) Mission Bay floating dock, CA, 28 Jan. 2007, photo J Byrnes; C) Otsuchi Bay aquaculture cage, Japan, 1 June 2007, photo T Otake; D) Surface pattern of two *Didemnum pardum* co-type specimens; E) MA Maritime Academy floating dock, 25 July 2007; F) Georges Bank, 45m, 1 Nov. 2003, photo D Blackwood; G) Sandwich tidepool, MA, 0.5m at low tide, 4 Dec. 2003, photo D Blackwood; H) Shilshole Marina subtidal cement wall, Seattle, WA March 2007, photo J Nichols; I) 1926 Mutsu Bay, Oka collection. Scale bars: A, F-G, 2 cm; B, I, 1 cm; C, E, H, 5 cm; D, 4 mm.



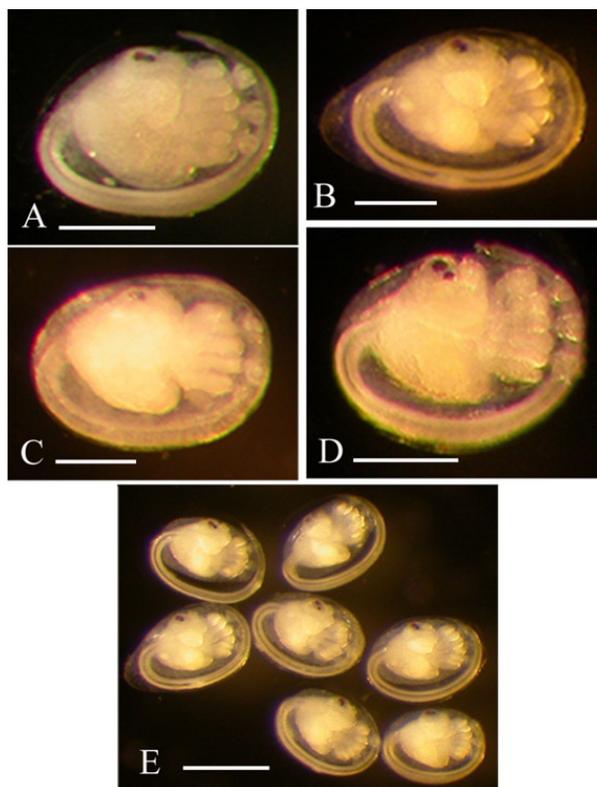
**Figure 2.** Tunic surface pattern of *Didemnum* sp. A colonies. A) Brest port floats, France, 28 Aug. 2005; B) Edmonds Underwater Park, WA, 26 Sept. 2004, on sunken boat hull, 10 m; C) Marine Biological Laboratory floating dock, MA, 24 July 2007; D) Asamushi, Mutsu Bay aquaculture cage, Japan, 13 July 2007, photo R Kuraishi; E) Perros Guirec floating dock, France, July 2002, photo S Maslakova; F) subtidal, Agamemnon Channel, British Columbia, 6-12 m, Dec. 2004, photo B Hanby. Scale bars: A-B, D-F 1 cm; C, 2 cm.



**Figure 3.** Spicules (SEM) from various populations of U.S. and Canadian *Didemnum* sp. A. All except C and F collected from floating docks. A) Woods Hole, MA; B) Newcastle, NH; C) Georges Bank, MA, 45 m; D) Poulsbo, WA; E) Des Moines, WA; F) Nanoose Bay, Vancouver Is., Canada, 30 m on kelp; G) Bodega Bay, CA; H) Malahide, Ireland. Scale bars: A, D, E, F, H, 20 µm; B, 25 µm; C, 30 µm; G, 40 µm.



**Figure 4.** A-F, H: Spicules (SEM) from various populations of European, Japanese and New Zealand *Didemnum* sp. A; G: *Didemnum* sp. B from Doubtful Sound, New Zealand. A) Misaki Marine Lab, Japan, floating dock; B) Otsuchi Bay, Japan, aquaculture cage; C) Asamushi Marine Lab, Japan, aquaculture cage; D) Ise Bay, Japan, low intertidal on rocks; E) New Zealand mussel line; F) Le Havre, France floating dock; H) Brest port, France, floating dock. Scale bars: A, 50  $\mu\text{m}$ ; B, D, E, G, H, 20  $\mu\text{m}$ ; C, F, 30  $\mu\text{m}$ .



**Figure 5.** A) Brooded unhatched *D. vexillum* larva, Whangamata, New Zealand. B-E, brooded unhatched *Didemnum* sp. A larvae. B) Le Havre, France; C) Sausalito, CA; D, Asamushi, Japan; E) Le Havre group of 7 larvae showing size variation within a single colony. Scale bars: A-D, 200  $\mu$ m; E, 400  $\mu$ m.

variation in both size and shape typical for this species. Figure 4G shows spicules from a different unidentified species, *Didemnum* sp. B from Doubtful Sound, New Zealand, collected subtidally on hydroids and debris. Its color is tan, it forms long lobes like *D. vexillum* and the spicules are similar but its larva has only 4 pairs of lateral ampullae and the DNA sequences of both a nuclear and mitochondrial gene are significantly different (Stefaniak et al. 2009, this issue). Typical examples of unhatched late-stage brooded tadpoles are compared in Figure 5. Figure 5E shows an average size range (506-572  $\mu$ m) of apparently mature unhatched tadpoles from a single colony. Tadpoles from all other *Didemnum* sp. A colonies collected worldwide (not shown) closely match those shown in Figure 5 in morphology and size range. Table 1 compares morphological characters of *Didemnum* sp. A with *D. vexillum* and several other described *Didemnum* species; see Discussion for explanation.

## Discussion

### Europe

*Didemnum* sp. A was not recognized as a non-native in various parts of Europe when first noticed during the 1990's; it was originally thought to be the proliferation of a native species and was identified by some as *Didemnum helgolandicum* and by others as *D. lahillei*. This is not surprising because the descriptions of these species are very confused (Carlisle 1954; Lafargue 1968, 1972, 1975) even in several of the most widely used identification guides for Europe (Millar 1966, 1970; Hayward and Ryland 1990). During an examination of the type specimen of *D. helgolandicum* in 2005, kindly loaned from the Hamburg museum, I luckily found brooded larvae, which Michaelsen (1921) missed when he described the species. The larvae have only 2 adhesive papillae and thus the invasive *Didemnum* sp. A which has 3 larval

**Table 1.** A comparison of morphological characters among several *Didemnum* species

Taxon	<i>D. sp. A</i> (all colonies worldwide)	<i>D. vexillum</i> Kott, 2002	<i>D. vestum</i> Kott, 2004	<i>D. lutarium</i> Van Name, 1910	<i>D. carnulentum</i> Ritter & Forsyth, 1917	<i>D. lahillei</i> of Lafargue 1968
Colony color	beige, cream, or pale orange	yellowish cream	pale yellow	white, yellow or pinkish	pale pink to white	beige or dirty yellow
Spicule-free dark bands between zooid groups	present	present	present	not in description	present	present
Colony shape	thin to thick, encrusting to lobed	thin to thick, encrusting to lobed	thin to thick, encrusting to lobed	2-4+ mm thick, wrinkled	thin (4 mm or less), encrusting	irregular encrusting or lobate, 1mm+ thick
Hypoabdominal lacunae	absent	absent	absent	absent	absent	absent
Zooid color	white thorax, yellow abdomen	white thorax, yellow abdomen	yellow	not in description	orange	not in description
Spicule density	sparse, mostly limited to surface	sparse, mostly limited to surface	sparse, limited to surface layer	dense, throughout tunic	mostly in upper layer of tunic	sparse, mostly limited to surface
Spicule shape, # of rays in optical transverse section	stellate, 8-11 rays (occasionally more)	stellate, 9-11 rays	stellate, 5-9 rays	stellate, usually 8 or 9 (based on illustration)	stellate, ~ 6-9 from figure	short stellate or burr-like
Spicule size	up to 57 $\mu\text{m}$ (average about 30)	up to 58 $\mu\text{m}$ (average 30 $\mu\text{m}$ in photo)	to 35 $\mu\text{m}$	most < 20 $\mu\text{m}$	20-75 $\mu\text{m}$	30 $\mu\text{m}$ average
# of stigmata per side in first row	8	8 or 9	6 (Kott 2004); actual count 8 in mature zooids	10-11 in rows 1-3	"about 6"	7
# of testis lobes	1	1	1	1	1	1
# of coils of sperm duct	8 to 11	9 (no range given)	8	8 or 9	6	8 to 9
# of pairs of larval lateral ampullae	6	6	not in description; no larvae in pieces examined	not in description	not in description	6
# of larval adhesive papillae	3	3	not in description	not in description	not in description	3
Size of mature unhatched larva	484-660 $\mu\text{m}$	600 $\mu\text{m}$ (no size range given)	not in description	not in description	not in description	up to 700 $\mu\text{m}$

Table 1 (continued)

Taxon	<i>D. lahillei</i> (Giard, 1872)	<i>D. pardum</i> Tokioka, 1962	<i>D. misakiense</i> Oka & Willey, 1892	<i>D. pacificum</i> Tokioka, 1953	<i>D. areolatum</i> Tokioka, 1953
Colony color	? tunic soft, gelatinous	yellowish orange surface in life; preserved colonies white w/ scattered red pigment granules	bright red	whitish, smooth, "frothy"	white with scattered red pigment granules
Spicule-free dark bands between zooid groups	absent	absent	absent	absent	absent
Colony shape	thin, encrusting	thin (0.5-1.0 mm), encrusting	thick, smooth, lobed	4-5 mm thick, lobed	thin (< 2 mm), encrusting
Hypoabdominal lacunae	absent	absent	present	present	absent
Zooid color	pale dirty yellow	brownish-black	white in preserved specimens	pale yellow	? zooids dead and decomposed
Spicule density	not in description	distributed evenly throughout tunic	mostly in upper layer of tunic	spicules absent	throughout tunic
Spicule shape, # of rays in optical transverse section	burr-like	Stellate	stellate	not in description	stellate, 8-11 rays in optical section
Spicule size	not in description	31-37 $\mu$ m	not in Oka & Willey; 13-18 $\mu$ m in Tokioka (1967)	not in description	up to 34 $\mu$ m
# of stigmata per side in first row	not in description	~6 in each row	not in descriptions	6 in first two rows, 5 in last two rows	not in description
# of testis lobes	1	1	1	1	not in description
# of coils of sperm duct	not in description	7 to 8	8 to 9	4	not in description
# of pairs of larval lateral ampullae	4	6	6	6	6
# of larval adhesive papillae	3	3	3	3	3
Size of mature unhatched larva	not in description	up to 590 $\mu$ m	not in descriptions	up to 560 $\mu$ m	up to 560 $\mu$ m; purplish brown

adhesive papillae could not be this species; this character, along with the spicule morphology and other characters, indicates that Michaelsen's *D. helgolandicum* is actually a junior synonym of *D. maculosum* (Milne Edwards, 1841), as had been concluded by F. Monniot (pers. comm.), Lafargue (1972, 1975) and Lafargue and Wahl (1987) in papers subsequent to Lafargue (1968).

The species we now know as *Didemnum lahillei* was originally described as *Leptoclinum gelatinosum* by Giard (1872). Hartmeyer (1909) changed the name to *Didemnum lahillei* after the genus *Leptoclinum* was synonymized under *Didemnum* because the species name *gelatinosum* then became preoccupied by a completely different species, *Didemnum gelatinosum* (Milne Edwards, 1841) (which was subsequently synonymized under *Diplosoma listerianum*!). Lafargue (1968) added greatly to the confusion by ignoring Giard's description and stating that *D. lahillei* tadpoles could have 5 or 6 pairs of lateral ampullae and illustrated a variety of spicule shapes both spined and burr-like, thus obviously lumping several species. Even in subsequent papers (Lafargue 1975, Lafargue and Wahl 1987) these errors remained. It is unfortunate that the specimens from the Glénan Archipelago, St. Vaast, Roscoff and other locations in France that Lafargue (1968), Médioni (1970) and Lafargue and Wahl (1987) identified as *D. helgolandicum* and *D. lahillei* cannot be located (F. Monniot pers. comm.). Their description of *D. lahillei* agrees in many (though not all) characters with *D. vexillum* (see Table 1) but is apparently a mixture of several species, as it combines characters of colonies collected from 4 locations including the Mediterranean: beige or pale yellow tunic with sparse spicules (though more burr-like in the drawings), narrow translucent spicule-free bands between groups of zooids, thorax with 7 stigmata in first row (8-9 given by Kott for *D. vexillum*), sperm duct with 8-9 coils, larva with 4 or 6 pairs of lateral ampullae. The specimens are thought to be at the Laboratoire Arago, Banyuls, but repeated inquiries from myself and F. Monniot have gone unanswered.

In December 2004, F. Monniot, X. Turon and E. Vázquez (pers. comm.) determined that the Mediterranean and Spanish Atlantic specimens of *D. lahillei* agree with Giard's (1872) original description of *L. gelatinosum* (the species that was changed to *D. lahillei* by Hartmeyer). The invasive *Didemnum* sp. A (which is a cool-water

species and has not been reported from the Mediterranean or Spanish Atlantic) varies considerably from Giard's *D. lahillei* in spicule shape (long pointed spines in *Didemnum* sp. A as shown in Figures 3 and 4, and burr-like in *D. lahillei*) and number of larval lateral ampullae (4 pairs in *D. lahillei*, 6 pairs in *Didemnum* sp. A).

Perhaps the first European report of *Didemnum* sp. A was in the Netherlands by Ates (1998) who listed it incorrectly as a range extension of *D. lahillei* and indicated that the first record in the Netherlands was 1991. "In 1996 and 1997 the species was extremely dominant in the Zijpe, part of the Eastern Scheldt estuary, where it covered an estimated 80 to 90% of the suitable substrate to the detriment of much of the original benthic fauna. The cause for the expansion of *D. lahillei* into the Eastern Scheldt is unknown and so is the cause for its local dominance in the Zijpe." In July and August 2002 colonies were found on floating docks at two small harbors in northwestern France: Perros Guirec and Camaret-sur-Mer (G. Lambert unpublished) and identified as *D. lahillei* by F. Monniot (pers. comm.) who also identified samples of the same species from Le Havre for G. Breton in early 2004. According to G. Breton, "the first time *Didemnum lahillei* [now known not to be that species] was found in the port of Le Havre was 13th December 1998, with a small but dense population in the south western part of the Bassin Vauban." In succeeding years it spread to other basins and has remained common (Breton 2005). During August and September 2005, on a rapid assessment survey (RAS) of numerous ports and marinas in Brittany for invasive ascidians, *Didemnum* sp. A was abundant at Le Havre as well as Brest and Concarneau and is still present at Perros Guirec (M. Nydam pers. comm.) and Camaret-sur-Mer (T. Honegger pers. comm.). New French records include Archachon, La Rochelle, and Pornic on the Atlantic side and Granville on the Channel (M. Nydam pers. comm. July 2007).

The first report of *Didemnum* sp. A in Ireland was October 2005 at Malahide Marina (Minchin and Sides 2006), but the extensive fouling indicated that it was probably not newly arrived. It was reported from a marina in Carlingford Lough, Ireland, in 2006 (Minchin and Sides 2006; Minchin 2007). Surprisingly, during a RAS of 12 sites in southern England from Brighton to Plymouth in September 2004 (Arenas et al. 2006) and further sampling of

several of the same sites in July 2007 (M. Nydam pers. comm.), no *Didemnum* sp. A was found. None was found during a RAS of 10 Scottish marinas in 2006 (Ashton et al. 2006). A search of numerous older publications on the marine invertebrates of the UK did not turn up any records of a *Didemnum* species resembling *Didemnum* sp. A (Berrill 1928; Thompson 1934; Kott 1952; Eales 1961; Picton 1985). It is impossible to know what species Millar's description (1966, 1970) of his obviously mis-identified *D. helgolandicum* actually refers to. Carlisle (1954) similarly confounded the descriptions of several *Didemnum* species.

#### United States

Massive colonies were observed at several sites in Massachusetts and Rhode Island during a RAS for invasive species in August 2000 (see table in Bullard et al. 2007a) and initially identified by me as *Didemnum lutarium* Van Name, 1910, because it seemed to agree with at least some aspects of the published description; that species had been reported to be very abundant and widespread in the Woods Hole region and other parts of New England according to Verrill and Smith (1874), Verrill and Rathbun (1879), Sumner et al (1913) and Van Name (1910, 1945), not only on natural subtidal substrates but also fouling pilings and other man-made structures. However, examination of a specimen of *D. lutarium* borrowed from the Smithsonian Institution National Museum of Natural History in 2002, in addition to a careful comparison of morphological characters (see Table 1), show that *Didemnum* sp. A is not *D. lutarium*. It is not clear just what *D. lutarium* is; the zooids "frequently, if not invariably" have 2 testes according to Van Name (1945). *D. albidum* (Verrill, 1871), an abundant New England species, always has 2 testes but has fewer sperm duct coils (4-8 listed by Van Name but only 4 -5 in all samples examined by me), much larger distinctive spicules with bluntly rounded rays (small pointed rays in *D. lutarium*) and is always white. Unfortunately there is no description or drawing of the larva, which taxonomists now realize provides crucial species-specific characters such as number of adhesive papillae and lateral ampullae, especially important for distinguishing *Didemnum* species. A specimen from the Gulf of St. Lawrence labeled *Didemnum candidum lutarium* borrowed from the Huntsman Marine Science Centre, St. Andrews, NB,

Canada is also not *Didemnum* sp. A but it is not clear what the species is. The colony contains larvae with about 20 lateral ampullae but the zooids have no testis or sperm duct, and the spicules do not match the description for *D. lutarium*. It is puzzling that *D. lutarium* was reported as abundant and widespread in New England between the 1870's and early 1900's, and now there is apparently no didemnid that matches its description. *D. albidum* is still common in northern New England and areas of the Georges Bank (unpublished observations; J. Collie pers. comm.).

The situation became much more complex when a sample from Coast Guard pier 3M, Portsmouth Harbor, Newcastle, New Hampshire was sent to Biosecurity New Zealand in 2002 for comparison with *D. vexillum*. The sample was forwarded to P. Kott who described it as a new species *D. vestum* (Kott 2004), claiming it was significantly different from her newly described *D. vexillum* (see below, New Zealand section). However, the morphological characters available in this incomplete description are all overlapping with those from numerous collections of *Didemnum* sp. A made by me and others from the type location and elsewhere worldwide between 2000 and 2007 (Table 1), and the DNA sequences of all samples from the type location of *D. vestum* are virtually the same as the other worldwide populations of *Didemnum* sp. A (Stefaniak et al. 2009, this issue). I was able to borrow three small pieces of the *D. vestum* holotype from the Queensland Museum, and all characters agree with the description of *D. vexillum* as well as all worldwide samples of *Didemnum* sp. A. Significantly, there were no *Didemnum* species reported from the *D. vestum* holotype location and a second site at Beverly, MA in a long-term monitoring study on invasive species from 1970 to 1992 (Berman et al. 1992); the first record of *Didemnum* sp. A at Portsmouth Harbor, NH is mid-January 2001 (Bullard et al. 2007a) by L. Harris, a marine ecologist who has been monitoring numerous sites in the Gulf of Maine including the *D. vestum* type location for over 30 years. He stated that "It is definitely new to our region" (pers. comm. 2001). In Kott's holotype the spicules are in poor condition and additionally were not well prepared for SEM (Kott 2004, Figure 1). Only two spicules in the figure can be seen at all, and those are partially obscured. Kott states that the poor spicular condition is probably "an artefact resulting from fixation and/or preservation". Kott mentions that

there are fewer spicules in the *D. vestum* holotype than in *D. vexillum*. This is a character that varies considerably between colonies and even between different regions of the same colony in very large colonies. Spicule size and abundance is greatly affected by salinity; during periods of heavy rainfall and lowered salinity the colonies can become almost aspiculate and those that do form are smaller than average (J. Dijkstra, G. Lambert unpub. obs.). Repeated collections over several years from the *D. vestum* holotype location and from numerous locations in the Pacific northwest during seasons when salinity is 30 ‰ or less due to heavy rainfall always have small sparse spicules, though DNA sequencing has shown that they are genetically the same species (Stefaniak et al. 2009 this issue). Salinity readings at the *D. vestum* holotype location were only 26-31 ‰ for June 2002 and <30 ‰ for all of July (J. Dijkstra pers. comm.); the holotype was collected in July 2002.

In the original description of *D. vestum* “The contracted thoraces obscure the actual number” of stigmata (Kott 2004); only in buds did she count 6 stigmata in the first row, but these were not fully formed adult zooids. Using a hematoxylin stain on a few isolated thoraces of mature zooids from the *D. vestum* holotype, I was able to count 8 stigmata per side in row 1 on several thoraces, the same as *D. vexillum* (Table 1). There is no description of the larva (nor did I find any in the holotype pieces I examined), a crucial character for distinguishing species in this genus. There is a single testis with 8 sperm duct coils listed by Kott; no intra-colony range in number of coils is given though in the hundreds of colonies of *Didemnum* sp. A that I have examined including colonies from the type locality, this character always varies within every colony between zooids and ranges from 8-11. I was able to count 8 sperm duct coils in a few hematoxylin-stained zooid abdomens from the holotype; the testes and sperm ducts were mostly empty of sperm and in an early stage of maturation. The colony color and morphology, color of zooids, eggs and larvae from other colonies from the type locality are the same as *D. vexillum*. Kott (2002) wrongly ascribes to me (via a third party [J. Culbertson] without verification by me) the incorrect claim that *D. perlucidum* F. Monniot, 1983 from the Gulf of Mexico has a larva with 6 pairs of lateral ampullae and thus could possibly be this *Didemnum* sp. A. I have examined hundreds of *D. perlucidum* colonies from many parts of the

world including the Texas coast collected by J. Culbertson and from S. Padre Island collected by myself (Lambert et al. 2005); it is a warm-water subtropical species (whose origin remains unknown) that has invaded many regions of the Atlantic and Pacific including the Texas coast but is quite different from *Didemnum* sp. A in many relevant morphological characters including the larva with 4 pairs of lateral ampullae (Monniot 1983). J. Culbertson did not send me any samples matching the description given by Kott.

The first verified record of *Didemnum* sp. A on the U.S. east coast (Damariscotta River estuary) is July 1993 (voucher specimen at Darling Marine Center, Maine, examined by me), but P. Yund and T. Miller (pers. comm.) saw it there as a fouling species in 1988, and there is photographic evidence of its presence in “high abundances” on oyster aquaculture nets in 1982 (Dijkstra et al. 2007a). Oystermen remember seeing *Didemnum* sp. A in the Damariscotta region during the 1970's, but “it only became a real pest by the beginning of the 1980's when it was so bad they had to go to bottom culture” (R. Clime, L. Harris pers. comm.). It was discovered on Georges Bank over a 16 km<sup>2</sup> area during a November 2003 photographic survey by remote operated vehicle (ROV) from NOAA ship Delaware II (J. Collie pers. comm.). A reexamination of earlier Georges Bank photos showed its presence as early as 1998, and on nearby Tillies Bank in 1996 (Bullard et al. 2007a). Subsequent surveys have expanded the known extent of *Didemnum* sp. A on Georges Bank to over 230 km<sup>2</sup> (Valentine et al. 2007a). Verrill (1884, 1885) does not list any *Didemnum* species records from the New England offshore banks. There are no records of *Didemnum* sp. A yet from the Canadian east coast, but since its first record in August 2004 at Eastport, Maine, at the Canadian border, it has spread and increased dramatically in abundance there (L. Harris pers. comm.); mussel growers and personnel with Fisheries and Oceans Canada are conducting frequent surveys for it.

On the U.S. west coast, in May 2003 I observed large colonies on floating docks in San Francisco, Humboldt and Tomales Bays. Samples collected by J. Byrnes and J. Stachowicz from the subtidal breakwater at the entrance to Humboldt Bay in northern California matched morphologically the populations fouling many east coast New England sites. Recent DNA sequencing of these samples confirmed a close

match to east coast and other sites (Stefaniak et al. 2009, this issue). Surprisingly no specimens were recorded during a RAS of San Francisco Bay in October 1997, though I determined that voucher samples collected by A. Cohen in 1993 (the earliest California record so far) were this species. Heavy rains during spring 1997 may have killed many of the colonies, but by 2003 the species was again abundant in San Francisco Bay. During the previous several years I had identified a number of didemnids from California harbor collections for various government agencies as the California native *D. carnulentum* Ritter and Forsyth, 1917 (Lambert and Lambert 1998) based on the original description because I have been unable to locate the type specimen; a reexamination of these samples indicated that they were in fact the new invader *Didemnum* sp. A. In addition to the California records of October 1993 for San Francisco Bay (3 locations), and November 1996 for Bahia Pt., Mission Bay, San Diego (one site, but recorded at 4 sites spring 1997) (G. and C. Lambert unpub. obs.), additional recently recorded California sites are listed in Bullard et al. (2007a). In January 2007 large abundant colonies were observed at Bahia Belle boat dock, Mission Bay, close to Bahia Pt.: “80-90% cover... I surveyed this area in August of 2003, and did not find any” (J. Byrnes pers. comm.). A reexamination of records showed that while the species was present at several sites in Mission Bay from Nov. 1996-May 1998 (but misidentified; see above), it was never recorded from the single site surveyed in Mission Bay during the RAS of August 2000 (Cohen et al. 2005), though this site had been surveyed several times since 1994 (Lambert and Lambert 2003). *Didemnum* sp. A has never been recorded from San Diego Bay. Thus its transience at any one site has been an important factor in the incomplete compilation of records and dating of its arrival in California. I examined a number of California *D. carnulentum* samples borrowed from the National Museum of Natural History, most of them collected from offshore islands in the 1970's, and none is *Didemnum* sp. A.

The first verified record in Washington state was October 1998 at the end of a long rope attached to floating docks in the west central region of Puget Sound at Poulsbo Yacht Club (misidentified by me as *D. carnulentum*). None was found during the 1998 Puget Sound RAS (Cohen et al. 1998). The next record is May 2000, as a common fouler of mussel strings and

rafts at Taylor Shellfish Co. in Totten Inlet, in south Puget Sound (Cohen et al. 2001, again misidentified by me as *D. carnulentum*), where it had apparently been present for an indeterminate number of years but was not a new arrival (G. King pers. comm.). It was documented there again in November 2004. It was found on a sunken wooden boat at an underwater park in Edmonds just north of Seattle in November 2003. Between its discovery and its removal in October 2004 the colony or colonies had increased from ~1 m to ~3.3 m in diameter (K. Frick pers. comm.). A single small colony was found in November 2004 at Des Moines marina just south of Seattle after a thorough search (Bullard et al. 2007a). Since then it has become much more abundant and widespread throughout the marina, and has been documented at a number of other sites in Puget Sound (Lambert 2006).

#### *British Columbia*

The first documented record in British Columbia is in 2003, heavily fouling mussel cages in Okeover Inlet, Malaspina Peninsula (49°58.8'N, 124°41.4'W). Numerous locations have been documented since then (identification confirmed by me), all at or near oyster farms (*Crassostrea gigas*, originally imported from Japan) including sightings by recreational divers in subtidal areas where it had not previously been seen, indicating that it is spreading rapidly and in some cases covering many square meters (Bullard et al. 2007a). Follow-up dives are confirming the rapid spread of this species (S. Geerlofs, S. Kurowski, A. Lamb, F. Poole pers. comm.), while anecdotal evidence from aquaculturists goes back to about 1991 (D. Paltzat pers. comm.). Most of the known localities so far, with photos, are posted on the US Geological Survey website (2008).

#### *New Zealand*

The only southern hemisphere country to report *Didemnum vexillum* so far is New Zealand. Colonies were first observed on the North Island in Tauranga Harbour in May 2001 and Whangamata Harbour in September or October 2001 (B. Coffey pers. comm.). In December 2001, huge colonies with long lobes (> 1 m) were found covering the hull of a barge that had been anchored for ~8 months in Shakespeare Bay, South Island (A. Coutts pers. comm.). The barge had a complicated history of movements around

New Zealand from 1992 until January 2001 when it was towed from Tauranga (thought to be the initial source of fouling) to Shakespeare Bay (Coutts 2002; Coutts and Forrest 2007). I received a sample from B. Coffey collected at Whangamata Harbour on 18 January 2002 and noted to him that it closely resembled the New England colonies collected in August 2000. B. Coffey also sent a sample from the same Whangamata Harbour location, collected on the same day, to P. Kott in Australia for identification which she promptly described as a new species *Didemnum vexillum* (Kott 2002), declaring it an overlooked native of New Zealand even though it exhibited all 10 criteria for designation as an invasive species (Chapman and Carlton 1991). *D. vexillum* was documented in Auckland's Viaduct Harbour in December 2005 and continues to spread to mussel and salmon farms and harbors throughout the Marlborough Sounds (A. Coutts pers. comm., Coutts and Forrest 2007). Efforts to control or eradicate it have not been successful. *Didemnum vexillum* is undoubtedly a very recent introduction to New Zealand. No *Didemnum* with a matching description was reported by Millar (1982) who included all New Zealand species described to that date. Skerman (1960) also does not include any *Didemnum* species. DNA sequences of two nuclear genes and the mitochondrial *col* gene from *D. vexillum* closely match the sequences from all other worldwide populations (Stefaniak et al. 2009, this issue). Thus the species is undoubtedly not native to New Zealand, where it was first observed on harbor structures in May 2001 and described from a sample collected in January 2002, but its region of origin remains unresolved.

The natural products chemistry of *Didemnum* sp. A samples from several locations worldwide was analyzed at the University of Auckland Dept. of Chemistry and compared with *D. vexillum* to determine if there were any biochemical similarities between the populations. All the samples are similar in that they "have very little evidence of secondary metabolite biosynthesis as judged by HPLC, MS and NMR analysis. There was no evidence for chemistry that is of interest to the marine chemistry fraternity or of use in drug discovery projects. The chemistry identified in all specimens includes adenosine and fatty acids. Both classes of compounds are very well represented in ascidians both in NZ waters and overseas" (B. Copp and T. Grkovic pers. comm.). A number of *Didemnum* species are

known to have extremely potent antibacterial, antiviral, and anti-cell cycle secondary metabolites (Prado et al. 2004 for example), some of which are species-specific, so the lack of any such compounds in all *Didemnum* sp. A and *D. vexillum* samples is of some interest.

There are as yet no records from Australia, South Africa or South America. M. Rius (pers. comm.) recently completed a survey of many sites around S. Africa for introduced ascidians and did not find it.

### Japan

It had been determined from numerous collections that *Didemnum* sp. A is a cool-water temperate species with a wide temperature range of about 0-28°C. (Bullard et al. 2007a, Valentine et al. 2007b, G. Lambert unpub. obs.), though with considerable die-back at the low end of this temperature range. Was there any cool temperate area of the world where *Didemnum* sp. A was present but had not been reported as a recent invader, indeed where it was known to have been present for many years and hopefully was already described? The answer might be Japan. Nishikawa (1990) collected similar colonies from various sites in northern Japan during the 1980's, including Otsuchi and Mutsu Bays and from buoys and net cages off the Oga Peninsula near Ise Bay. He compared these samples, as well as a 1926 museum sample from Mutsu Bay (labeled only as *Didemnum* sp.), with the 4 known Japanese *Didemnum* spp. that have larvae with 6 pairs of lateral ampullae and concluded that these specimens probably were *Didemnum pardum*, described from Sagami Bay by Tokioka (1962). Nishikawa (1990) made his determination with some reservations, listing certain morphological differences from *D. pardum* which in light of recent findings indicate that his samples are not *D. pardum* but most likely are conspecific with *Didemnum* sp. A (see Table 1 for a comparison of the 4 relevant Japanese species with *Didemnum* sp. A). In July 2005 Nishikawa (pers. comm.) examined 4 syntypes of *D. pardum* and described them as follows: "The type material, registered as NSMT-PcR 315b, consists of only 4 small colonies, although Tokioka's original description [indicates] 'many colonies'. These 4 syntype colonies are white in color, dark coloration of zooids mentioned in the description is not detected. Zooids are more or less deteriorated, and include no larvae. Unfortunately the detailed structure of zooids is

hard to examine, but the absence of 'hypoabdominal lacunae' in the description can be confirmed. Spicules were examined by SEM, and the two forms of spicules, with 'blunt' and 'bluntly pointed' rays in the description were detected. However, the 'blunt' form may be due to a decay of 'bluntly pointed' form, caused probably by formalin as the fixative, judging from the SEM images."

I was able to examine two of these small syntype *D. pardum* colonies of NSMT-PcR 315b, from the Showa Memorial Institute, National Museum of Nature and Science, Tsukuba City, Japan and my observations agree with those of Dr. Nishikawa. Very significantly, the tunic of these colonies does not exhibit the characteristic meandering dark lines that indicate the thoracic lacuna system, where there are no or few spicules in *Didemnum* sp. A (compare Figure 1D with Figure 1A-C, E-H and Figure 2). In *D. pardum* the "spicules are distributed evenly throughout the test from the surface to the bottom" (Tokioka 1962). In all *Didemnum* sp. A, the spicules are sparse and mostly confined to the upper layer of tunic. Most of the *D. pardum* spicules have bluntly rounded rays and I believe that this may be a species trait not a result of having become partially dissolved; less abundant slightly more pointed spicules are mixed in with and adjacent to them. *Didemnum pardum* zooids were described by Tokioka (1962) as "dark purplish brown or brownish black" in life easily seen through the pale tunic as "observed by members of the biological Laboratory of the Imperial Household", giving the colony a leopard-like appearance and thus the reason for Tokioka's choice of species name. Although the preserved zooids are not colored, nor were they when Tokioka examined the fixed material, there are numerous dark red pigment granules scattered throughout the tunic and especially in the basal layer (a feature mentioned by Tokioka 1962), a possible indication that the zooids were pigmented in life but the pigment dissipated after fixation, as sometimes happens. The zooids in all the worldwide populations of *Didemnum* sp. A are either colorless or pale yellowish-orange and there are no similar pigment granules in the tunic.

The 1926 Mutsu Bay sample of *Didemnum* sp. (see Methods) was generously loaned to me from the National Science Museum, Tokyo. The tunic is a pale yellow and has the meandering dark spicule-free lines that indicate the cloacal canal system (Figure 1I), exactly like all the

*Didemnum* sp. A colonies (compare with Figure 2) and reported by Nishikawa (1990). The spicules are sparse though somewhat denser than in most *Didemnum* sp. A colonies but the distribution is similar; they are mostly confined to the surface with few in the tunic matrix, in contrast to Nishikawa's statement that the spicules are dense and evenly distributed throughout the tunic. In these colonies there are also sparsely distributed spicules in the basal layer. The spicules are the same size and shape range as in all other *Didemnum* sp. A samples. Nishikawa (1990) measured the spicules as 10-30  $\mu\text{m}$  in diameter; I found a few up to 44  $\mu\text{m}$ . Unfortunately the zooids and larvae have disintegrated. They were in good condition when Nishikawa examined them; he observed that there are 8 stigmata per side in the branchial sac, the sperm duct coils 8-11 times around the single testis and the most advanced larvae, ranging in size from 500-625  $\mu\text{m}$ , have 6 pairs of lateral ampullae (Nishikawa 1990), all characters matching *Didemnum* sp. A.

The other three described Japanese species of interest, because they all have larvae with 6 pairs of lateral ampullae, are *D. misakiense* (Oka and Willey, 1892); *D. areolatum* Tokioka, 1953; and *D. pacificum* Tokioka, 1953. The colony of *D. misakiense* borrowed from the National Museum of Natural History, Washington DC had been identified by Tokioka (1967) who had also examined Oka and Willey's type specimen (Tokioka 1955); I could easily confirm that *Didemnum* sp. A is not this species. The colony still exhibits remnants of the brilliant red color so carefully described and illustrated by Oka and Willey (1892), and the smooth, uniform tunic composition, arrangement of zooids, presence of hypoabdominal lacunae and other characters (Table 1) also easily distinguish it from *Didemnum* sp. A.

I was fortunately able to examine the type specimens of *D. pacificum* and *D. areolatum*, borrowed from the Showa Memorial Institute, National Museum of Nature and Science, Tsukuba City, Japan. See Table 1 for a comparison of a number of morphological characters with *Didemnum* sp. A and other relevant species. Unlike *Didemnum* sp. A, neither species has the meandering dark lines visible on the tunic surface where spicules are sparse that indicate the thoracic lacuna system. *D. pacificum* has spacious hypoabdominal lacunae and struts of tunic through which the embryos travel from the posterior end of the zooidal abdomens to the

basal tunic layer. There are only 4 sperm duct coils (as described by Tokioka 1953; I was not able to find any zooids with a sperm duct though a few have a small testis forming). There are no spicules in the tunic, and no spicular envelopes became visible after staining with 1% toluidine blue; this technique is usually successful with didemnid tunic in which the spicules have dissolved (unpub. obs.). In *D. areolatum* the spicules are dense throughout the colony except in the spicule-free superficial bladder cell layer, as described by Tokioka (1953). There are many purplish-brown or reddish pigment granules throughout the tunic as in *D. pardum*, and the larvae (but not the zooids) are pigmented purplish-brown. As in *D. pardum*, the zooids reside in pockets in the tunic, surrounded by dense spicules. There are several other significant similarities with *D. pardum* which suggests that *D. pardum* might possibly be a junior synonym of *D. areolatum*. Tokioka (1962) commented that *D. pardum* resembled *D. areolatum* with regard to the morphology and distribution of tunic spicules and larval morphology, but distinguished it from his previously described *D. areolatum* by repeating from the original description that “in *D. areolatum* the superficial layer of the test above the thoracic lacuna-system is quite devoid of spicules and this gives the colony an areolate appearance.” However, this bladder cell layer, though much thinner in the very small *D. pardum* syntypes, is present. In *D. areolatum* the areolate appearance is due to numerous sunken areas around the zooids and closely resembles *D. pardum* (Figure 1D); the spicules are not actually absent but can be seen in the sunken areas of tunic. The spicules of *D. areolatum* were originally described as “distributed evenly and densely throughout the test” (except over the lacuna-system). Tokioka did not describe the zooids of *D. areolatum*, listing them only as “dead and decomposed”. I did find a few zooids that were not completely decomposed; though morphological details like number of stigmata per row could not be determined, I could see that there was no developed testis or sperm duct. The many larvae were in good condition. In spite of the lack of information about the zooids, the other differences between *D. areolatum* and *Didemnum* sp. A are enough to say with confidence that they are not the same species.

The samples from Ise Bay collected July 2003 by T. Nishikawa, Otsuchi Bay collected in June 2007 by T. Otake, and Mutsu Bay collected by

G. Miller-Messner and R. Kuraishi in July 2007 and by M. Byrne in September 2007, are a perfect match morphologically with all the other worldwide *Didemnum* sp. A samples (Figures 1-5) and most importantly, the DNA sequences are a close match (Stefaniak et al. 2009, this issue). Samples from Misaki marine station collected by G. Miller-Messner July 2007 also match morphologically in all characters but the DNA has not yet been sequenced. I was fortunate to be able to examine a *Didemnum* sample collected from cultured oysters in Hiroshima Bay 27 March 2006 that was misidentified as *D. moseleyi* (Herdman, 1886); it has many morphological differences from the tropical *D. moseleyi* and is another exact morphological match with *Didemnum* sp. A though it lacks brooded larvae. *Didemnum* sp. A in Japan is thus currently known from Hiroshima Bay, Ise Bay, Otsuchi Bay, Mutsu Bay and Misaki marine station, all on the main island of Honshu.

### Conclusions

*Didemnum* sp. A does not match any of the described Japanese species but does match the descriptions of *Didemnum* samples collected by Nishikawa (1990) that he identified as *D. pardum*, including the 1926 Oka collection *Didemnum* sp. sample. The published description of *D. vexillum* as well as examination of a co-type of *D. vexillum* sent to me by B. Coffey (who collected it from the same location on the same day and who sent part of his sample to P. Kott that she used for her description; see discussion in New Zealand section), provides the closest match to all worldwide samples of *Didemnum* sp. A (Table 1). The only valid published description of this species is *Didemnum vexillum* Kott, 2002, and therefore this is the name that must be used even though the species is undoubtedly not native to New Zealand and is a very recent introduction to that country.

The name *Didemnum vestum* Kott, 2004, must be relegated to a junior synonym of *D. vexillum* based on the close morphological and genetic similarities. *Didemnum vexillum* is probably native to Japan; the earliest worldwide record we have is 1926 from Mutsu Bay, where it is still common. The possibility does exist that it might have been introduced to Japan by boat traffic from elsewhere, perhaps some other Asian country, long ago. It is widespread and common in Japan, especially as a fouler of cultured bivalves, net cages, and various other artificial

structures, though it can be found on natural benthic surfaces such as the rocky low intertidal of Ise Bay (T. Nishikawa pers. comm.); the 1926 sample was overgrowing *Zostera* and other materials that can still be seen. A much larger-scale study, involving the collection and DNA sequencing of hundreds of samples from Japan and elsewhere around the world, will be necessary to determine the haplotype variability, enabling a more definite decision about the origin of this species.

#### *Possible vectors*

The worldwide transporting and transplanting of various oyster species, and the concomitant introduction of a large assortment of associated invertebrate and algal foulers, has a long and complicated history (see reviews by Andrews 1980; Carlton and Mann 1996), but perhaps the most widely exported oyster species has been the Japanese oyster *Crassostrea gigas*. It is tempting to implicate the export of adults as well as *C. gigas* seed on shell from Japan to many of the countries that now have widespread and heavy fouling by *Didemnum vexillum* (France, U.S. and Canada west coast, New Zealand) (McMillan and Bonnot 1931; Marteil and Barrau 1972; Quayle 1988; Grizel and Héral 1991). After the decline of the slow-growing small native Olympia oyster *Ostrea lurida* in the Pacific Northwest, large shipments of *C. gigas* seed on oyster shell were exported from Miyagi prefecture for many years to British Columbia up to about 1960. Miyagi is an area of Japan where *D. vexillum* has been common at least since the early 1980's (identified as *D. pardum* by Nishikawa 1990, corrected by T. Nishikawa pers. comm. 2005). It was then and still is a significant fouler of cultivated oysters and other bivalves, ascidians, and fish farm net cages. Quayle (1969) reported "Tunicates... will seldom be found on bed oysters but may be quite numerous on raft culture oysters or on seed strings, particularly in Pendrell Sound [B.C.]". He does not say whether these were native or introduced ascidians and there is no indication of the particular species involved. However, in a subsequent publication (Quayle 1988), his Figure 94 illustrates five common ascidian foulers of oysters, none of which are didemnids; he does not mention any didemnids on oyster strings even into the 1980's though he does list other invertebrates introduced via the oyster seed. Oysters were grown primarily in intertidal beds until the 1980's, so it

is unlikely that *D. vexillum* gained a foothold on the stock until the various culture methods involving complete submersion such as rack and tray and longline became common. In British Columbia a great deal of movement of oyster strings continues, especially from Pendrell Sound, the center for oyster spawning in B.C., to numerous grow-out areas in the province. Nearly every oyster and mussel (*Mytilus galloprovincialis*) farm in B.C. is now heavily fouled by *D. vexillum*, even in remote areas (D. Paltzat pers. comm.). Anecdotal evidence by longtime employees of the presence of *D. vexillum* dates back to about 1991, most of the earlier employees having retired. Movement of cultured oyster and mussel lines is also common in Washington state. *D. vexillum* has been a common fouler of these cultured bivalves in southern Puget Sound for many years (G. King pers. comm., Cohen et al. 2000 as *D. carnulentum*), and movement of mussel strings from southern Puget Sound to grow-out at the north end of Hood Canal may be the reason *D. vexillum* fouls mussels and gear at the Hood Canal location. The recent rapid expansion of *D. vexillum* populations into many marinas in Washington (Lambert 2006) is probably due to recreational boat traffic, which may be a highly significant vector once a species has made a successful transoceanic transplantation (Wasson et al. 2001; Minchin et al. 2006).

In the late 1960's there was a rapid and economically disastrous die-off of the cultivated Portuguese oyster *C. angulata* in bays around Brittany and Atlantic France due to a viral disease. Huge quantities of *C. gigas* seed stock on shell were flown in and brought by ship from Japan until about 1977, mostly from Miyagi prefecture (Marteil and Barrau 1972; Gruet et al. 1976; Grizel and Héral 1991). Complicating the picture, large quantities of adult *C. gigas* brood stock were exported from British Columbia to France at least through 1975 (Grizel and Héral 1991) and were mainly checked only for disease and predators such as oyster drill and flatworms; it is not clear whether they were treated for fouling. Though not listed as a major fouler, Gruet et al. (1976) mention "a few" living unidentified didemnids on the Japanese spat collectors examined upon their arrival in France, along with several species of solitary ascidians and other foulers, even after two 1-hr immersions of the collectors in fresh water prior to implantation. Katayama and Ikeda (1987) found that *Didemnum moseleyi* fouling oysters

could survive 2 hrs or more of freshwater immersion or air drying at Ushimado Fisheries Research Center, depending on ambient temperature. Probably this species is actually *D. vexillum* based on the recent examination of the Hiroshima Bay *Didemnum* incorrectly labeled as *D. moseleyi* (see above). Exports of Japanese oysters made prior to 1970 were implanted into French waters without any treatments to kill foulers, but aside from Gruet et al. (1976) there are no reports of didemnids having been imported. *D. vexillum* is now common at nearly all the *C. gigas* culture locations along the Atlantic and English Channel coasts of France (G. Lambert unpub. obs., M. Nydam pers. comm.) but are presumed to have arrived by vectors other than oyster imports. Thus while *D. vexillum* might have been imported to France and the western U.S. and Canada from Japan via fouled imported oysters, there is no direct evidence for it. Interestingly, Arakawa (1990) lists the 7 major species of ascidian foulers of cultivated oysters identified at numerous sites in Japan in the 1960's in a survey by Mawatari (1967) and there are no *Didemnum* species included, though the total number of ascidian species Mawatari found was 116. However, Miyazaki (1938) details heavy fouling of cultured oysters at the Kanazawa Oyster Farm in Kanagawa Prefecture by ascidians including *Leptoclinum album*, a species now synonymized under *Didemnum moseleyi*. Thus this report of *L. album* may well be *D. vexillum*.

*D. vexillum* has been common in the northeast U.S. at least since the 1970's when it was observed as a fouler of the cultured eastern oyster *Crassostrea virginica* (R. Clime, L. Harris pers. comm.). *C. gigas* is not cultured on the U.S. east coast and was never officially imported into New England, though large shipments of Olympia oysters from the Pacific NW were. The eastern oyster was exported to California, Washington and British Columbia for a long time, and large quantities of Olympia oysters were shipped to the east coast and to California. There are virtually no records of the foulers that would have been shipped along with the oysters but also no comments about any significant didemnid fouling. Some countries that imported large quantities of Japanese oyster seed on shell for grow-out have yet to report the presence of *D. vexillum* (Australia, South Africa, Tasmania) but in these countries most oyster culture was intertidal with the oysters exposed to air part of

every tidal cycle (Arakawa 1990). Japanese oysters were first introduced to New Zealand many years ago but *D. vexillum* only appeared suddenly in 2001 (Coutts 2002, B. Coffey pers. comm.).

The probable trans-oceanic vector is thus shipping. While oysters (*Crassostrea gigas*) from Japan were transported to both the North American Pacific coast (up to the 1960s) and to Europe (in the 1960s and 1970s), these episodes ceased many years before *D. vexillum* appeared. The "temporal disconnect" (J.T. Carlton pers. comm.) between oyster imports and the appearance of *D. vexillum* eliminates this vector possibility. With the possible exception of the photographic evidence of *D. vexillum* in the Gulf of Maine in 1982 (and thus present some years earlier, in the 1970s), the global appearance of this ascidian in the 1990s in Europe (1991), the eastern Pacific (California in 1993, and possibly a few years earlier in British Columbia), and New Zealand (2001), is almost certainly related to ship-mediated transport. J.T. Carlton (pers. comm.) "thoroughly collected the float fouling fauna of marinas, ports, docks, etc., from Vancouver Island to central California on two extended expeditions in 1976 and in 1977, and *Didemnum* wasn't there." Long distance survival of didemnids fouling the hulls of fast-moving ships is unlikely, but they could survive on the hulls of slow-moving barges as is thought to have been a possible vector for *D. vexillum* (Coutts and Forrest 2007), regions of hulls where extensive fouling can develop in low-energy areas such as the propeller shaft housing (J.T. Carlton, pers. comm. 2008) and also in sea chests (Coutts and Dodgshun 2007; Lee and Chown 2007). Transport of ascidians from the northern to the southern hemisphere or vice versa, undoubtedly by shipping, has resulted in the successful establishment of several species (Brewin 1946; Lambert 2004, 2007; Rius et al. 2008). Fragments of *D. vexillum* could survive in ballast water; there is ample documentation that every piece of adult colony of *D. vexillum* is a potential propagule with a high capability of reattachment and growth (Bullard et al. 2007b, Coutts and Forrest 2007, Osman and Whitlatch 2007, Valentine et al. 2007a). Recently in New Zealand the movement of a fish farm net heavily fouled with *D. vexillum* to an uninfected mussel growing area resulted in extremely rapid large-scale fouling of the mussel lines within weeks (A. Coutts pers. comm.).

However it arrived in New England, *D. vexillum* may have been subsequently introduced offshore on Georges Bank via contaminated scallop dredging gear and boats from their home ports; dredging fragments the colonies which then are carried on currents until they settle, reattach and grow. Fragments are known to survive in suspension for more than four weeks (M. Carman pers. comm.). Evidence is accumulating that artificial structures may facilitate invasions (Lambert and Lambert 1998, 2003; Oren and Benayahu 1998; Glasby et al. 2007; Tyrell and Byers 2007), probably due to several factors: proximity to the location of introduction of propagules (usually harbors), reduced bio-diversity, limited access by potential predators especially on floating structures, and location in disturbed habitats which results in a rapid turnover of resident species and availability of space for colonization. Rapid regional and local dispersal can then result from many modes of transport, with slower moving recreational vessels and barges, moving between marinas, ports, and harbors, likely being one of the most significant vectors (Wasson et al. 2001); movement of cultured aquaculture products (such as mussels and oysters), and gear, is common, with often little or no effort made to remove fouling species.

#### *Ecological implications of the worldwide invasions and predictions of future spreading*

*Didemnum vexillum* can be considered an “ecosystem engineer” because it is capable of drastically adversely modifying the habitats it invades (Wallentinus and Nyberg 2007). It is an unusual species in that wherever it has been reported as a new introduction it grows extremely rapidly, quickly covering large areas, sometimes hundreds of square meters. This phenomenal growth rate results in massive colonies that overgrow almost every other sessile species (Coutts and Forrest 2007; Gittenberger 2007; Valentine et al. 2007a, b; L. Harris, A. Lamb pers. comm.). On suspended mussel lines, floating docks, boat hulls and other structures in quiet waters it quickly forms long fingerlike lobes that break off easily, float away, and are capable of reattachment and growth (Bullard et al. 2007b; Coutts and Forrest 2007; Valentine et al. 2007a). These fragments may likely contain brooded larvae capable of being released either during dispersal or after reattachment. Unlike some introduced species that remain restricted to

artificial substrates in harbors even many decades after their first documentation, *D. vexillum* can quickly colonize and overgrow apparently healthy natural subtidal benthic substrates (Dijkstra et al. 2007a, b; Osman and Whitlatch 2007; Valentine et al. 2007a, b). In addition, the reproductive season is long, with colonies releasing huge numbers of larvae over several months (P. Valentine, M. Carman unpub. obs.). On the Georges Bank *D. vexillum* now covers over 230 km<sup>2</sup> (Valentine et al. 2007a), and elsewhere such as British Columbia in the vicinity of oyster and mussel farms new benthic areas are becoming overgrown as this species continues to spread (see photos at US Geological Survey 2008 for B.C., Netherlands, and other regions). Studies are underway to determine the effects of this overgrowth on the native benthic community, and its effects on bottom fish on the Georges Bank. The species has become a significant pest for aquaculturists (Coutts and Forrest 2007, L. Harris, T. Therriault, D. Paltzat pers. comm.). Most of the new records have appeared in the past 10-15 years (Breton 2005; Bullard et al. 2007a; Coutts and Forrest 2007; Gittenberger 2007; Minchin and Sides 2006; Minchin 2007). Though it will be difficult to prove, some authors speculate that there could be a link between the recent increase in the number of invaded sites and increase in biomass at more long-established sites with gradual increases in eutrophication and gradual warming over this time period. Bak et al. (1998) found a direct correlation between large increases in population density of the bacterial suspension feeder *Trididemnum solidum* and bacterial level in the water around Curaçao over a 15 year period. Like some other invasive ascidians, *D. vexillum* may well be tolerant of occasional low levels of dissolved oxygen which gives them a competitive advantage (Jewett et al. 2005).

Few predators have been reported for *D. vexillum*, though photographic evidence (US Geological Survey 2008) is accumulating for predation by large sea stars and sea urchins (Bullard et al. 2007a; A. Coutts, B. Hanby, L. Harris, S. Kurowski, F. Poole pers. comm.); littorine snails feed avidly on dying colonies (Valentine et al. 2007b) and also live colonies (G. Lambert unpub. obs.). A chiton has been observed feeding on *D. vexillum* in New Zealand (A. Coutts pers. comm.). Interestingly, *D. vexillum* lacks the potent anti-predator secondary metabolites found in many other didemnid species (B. Copp pers. comm.).

*D. vexillum* shows no sign of dying out in areas it has successfully invaded, and new invasions continue to be reported; thus it is still spreading worldwide in cool temperate areas. Many of the invasions are recent, and their long-term effects are not yet known.

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