

Research Article

Distribution and population dynamics of key ascidians in North Carolina harbors and marinas

Stephanie M. Villalobos¹, Gretchen Lambert², Noa Shenkar³ and Susanna López-Legentil^{1,*}

¹*Department of Biology and Marine Biology and Center for Marine Science, University of North Carolina Wilmington, Wilmington, NC, 28409, USA*

²*University of Washington Friday Harbor Labs, Friday Harbor, WA 98250, USA*

³*Department of Zoology, and The Steinhardt Museum of Natural History, Israel National Center for Biodiversity Studies, Tel-Aviv University, Tel Aviv 69978, Israel*

*Corresponding author

E-mail: lopezlegentils@uncw.edu

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Abstract

Ascidians have successfully invaded marinas and harbors around the world. Despite broad knowledge of their global ranges, in some locations, including the state of North Carolina, U.S.A., little is known about the community composition and distribution of native and introduced ascidians. We conducted field surveys at 16 harbors and marinas along the coast of North Carolina (33–35 °N) and documented the diversity, distribution and relative abundance of all ascidian species. Ascidians were identified using morphological observations and barcode sequencing of the mitochondrial gene cytochrome *c* oxidase subunit I. Distribution patterns of native and introduced ascidians were analyzed using presence-absence and relative abundance matrices in relation to latitudinal position (South *versus* North) and geographic distance among harbors. Finally, we monitored the dynamics of a well-established ascidian community at Wilmington over 1.5 years using photo transects. For each ascidian species, we calculated percent cover and abundance and then related those values to temperature fluctuations using cross-correlation analyses. Overall, we found three introduced, two cryptogenic, and eight native ascidian species. Geographic location and distance between survey sites had no effect on ascidian community composition in terms of presence-absence of species. However, the relative number of individuals per species present at each harbor was significantly related to the distance between sites. The ascidian community at Wilmington (three native, one cryptogenic, and one introduced species) showed some seasonality, with abundance and/or percent cover significantly correlated with temperature values recorded during the same month or a few months beforehand.

Key words: Sea-squirt, tunicate, introduced, cryptogenic, United States, biological invasion

Introduction

Introduced species are prevalent around the world, especially in the marine environment. Some species have become invasive (Blackburn et al. 2011) and affected marine ecosystems by altering the existing species assemblages and disrupting ecological relationships (Molnar et al. 2008; Craig 2010). Human-mediated invasions in marine ecosystems are most prevalent (Ruiz et al. 1997), with increased worldwide shipping causing the spread of introduced species to increase exponentially (Seebens et al. 2013). Introduced species readily colonize altered habitats, such as those suffering from biodiversity declines caused by pollution, overfishing, etc. (Briggs 2010). In North

America, many coastal introduced species have been reported attached to hard substrates in relatively sheltered waters of bays and estuaries (Ruiz et al. 2009). Ascidians (Chordata; Tunicata), have been conspicuously introduced in bays and harbors where low wave action, available hard artificial substrate, and elevated levels of bacterial food sources provide them with an ideal habitat (Naranjo et al. 1996; Glasby and Connell 1999; Lambert 2005; Shenkar et al. 2008).

Shenkar and Swalla (2011) reported 64 globally introduced ascidian species, 50 of which were observed in temperate environments. More recently, Zhan et al. (2015) increased the number of globally introduced ascidians to 80 species based on an exhaustive literature review. Ascidians share introduction vectors

and pathways with other introduced species, and thus can serve as a model to provide insights into common introduction pathways and secondary routes of spread (Zhan et al. 2015).

The biology of ascidians makes them highly successful invasive species. Adult ascidians may be dispersed as a part of a fouling community on ships' hulls, in sea chests, and on aquaculture materials (Ruiz et al. 1997; Briggs 2007; Herborg et al. 2009; Frey et al. 2014; López-Legentil et al. 2015). Many ascidians have long breeding seasons, high reproductive capacities, and rapid population growth rates (Rocha 1991; Lambert 2005; Pineda et al. 2013). However, ascidians produce short-lived, non-feeding larvae that usually settle within a few hours or days (Svane and Young 1989; Lambert 2005). Thus, ascidians have a hard time reaching new geographic areas unless they are assisted by anthropogenic transport. Ascidians often tolerate a wide range of environmental conditions, including varying levels of salinity, temperature, and pollution (Lambert and Lambert 2003; Lambert 2005; Piola and Johnston 2007; Pineda et al. 2012a; Rajiman-Nagar and Shenkar 2016). Ascidians are often strong competitors for food and space and can outcompete native species (Yamaguchi 1975; Sutherland 1978; Stachowicz et al. 2002; Carver et al. 2003; Lambert and Lambert 2003).

Introduced ascidians are common in North America. Simkanin et al. (2016) reported 26 introduced ascidian species in the United States and Alaska, 14 of which were found on the Atlantic coast. However, sampling along the East U.S. coast did not include the state of North Carolina (NC). Coastal NC is characterized by a variety of anthropogenic and environmental conditions that may foster the spread and establishment of introduced species. The NC coast includes part of the Atlantic Intracoastal Waterway (AIW), which extends from Norfolk, Virginia to Miami, Florida. The waterway was built to provide a protected navigation channel for trade, and supports high levels of commercial and recreational traffic. NC is in relatively close proximity to the Chesapeake Bay (~130 km away), which hosts the fourth largest port in the United States (American Association of Port Authorities 2001; Drake et al. 2005). The coast of NC is also bathed by both the cool Virginian Coastal current flowing southward from Cape Cod, and the warmer waters of the Gulf Stream flowing north. The confluence of these two major oceanographic features allows for the existence of three biogeographic provinces in NC (Virginian, Carolinian, and Tropical), each of which is characterized by a different assemblage of species (Cerame-Vivas and Gray 1966). High national and international maritime traffic along the NC coast guarantees recurrent

introductions of established introduced species and the arrival of new ones. Recurrent introductions increase propagule pressure and thus the probability of a successful introduction over time (Dupont et al. 2010; Goldstein et al. 2011; Pineda et al. 2011; Rius et al. 2012). However, although the NC coastline supports heavy ship traffic, to date the spread and prevalence of introduced species in NC is largely unknown.

This study aimed to determine the diversity and distribution of ascidians in sixteen harbors and marinas along the NC coast and to assess the temporal dynamics of a representative ascidian community at one site. The three main objectives were: (1) describe the current diversity of ascidians in NC harbors and marinas using both morphological keys and genetic barcoding; (2) determine the distribution patterns of native, cryptogenic, and introduced ascidian species; and (3) characterize the temporal dynamics of a well-established community at Wilmington, NC, including shifts in species diversity and overall coverage.

Material and methods

Ascidian diversity in NC and distribution patterns

Sample collection and processing

Surveys were conducted at sixteen harbors and marinas along the North Carolina coast in July 2014 (Figure 1, Table 1). GPS coordinates, water temperature, and salinity at the time of sampling were recorded for each sampling location (Supplementary material Table S1). Surveys were conducted using a variant of the Rapid Assessment Method described by Campbell et al. (2007), with relative abundance estimated according to the number of individuals observed: (1) rare: one or few specimens of a species observed, (2) common: species frequently observed but in low numbers, (3) abundant: species occurring frequently in sizable numbers, and (4) very abundant: species occurring frequently and in great numbers or clusters of individuals (Table 2). One or two specimens of each ascidian species were photographed *in situ* then collected in Ziploc[®] bags filled with seawater from the marina and a few menthol crystals to relax the zooids (see below). All specimens were collected from 0 and 2 m below the surface, and were found on pilings, submersed docks, and buoys.

Samples were maintained in seawater with menthol crystals for at least 2 hours before processing. After this time, each ascidian was placed in a plastic tray filled with seawater, assigned a sample code, and photographed. For colonial species, a piece of each colony was dissected and placed in a 20 mL scintillation vial filled with 100% ethanol for DNA

Figure 1. Sampling locations in North Carolina, USA. Divided into latitudinal regions: northern sites (circles) and southern sites (squares). Sites are as follows: South Harbor Village Marina (A); Southport Marina (B); Harbour Point Marina (C); Joyner Marina (D); Inlet Watch Yacht Club (E); Masonboro Yacht Club & Marina (F); Seapath Yacht Club (G); Bridge Tender Marina (H); Wrightsville Beach Marina (I); Harbour Village Marina (J); Crow’s Nest Yacht Club (K); Portside Marina (L); Olde Towne Yacht Club (M); Harkers Island (N); Ocracoke Ferry Landing (O); Hatteras Harbor Marina (P) (for details see Supplementary material Table S1).

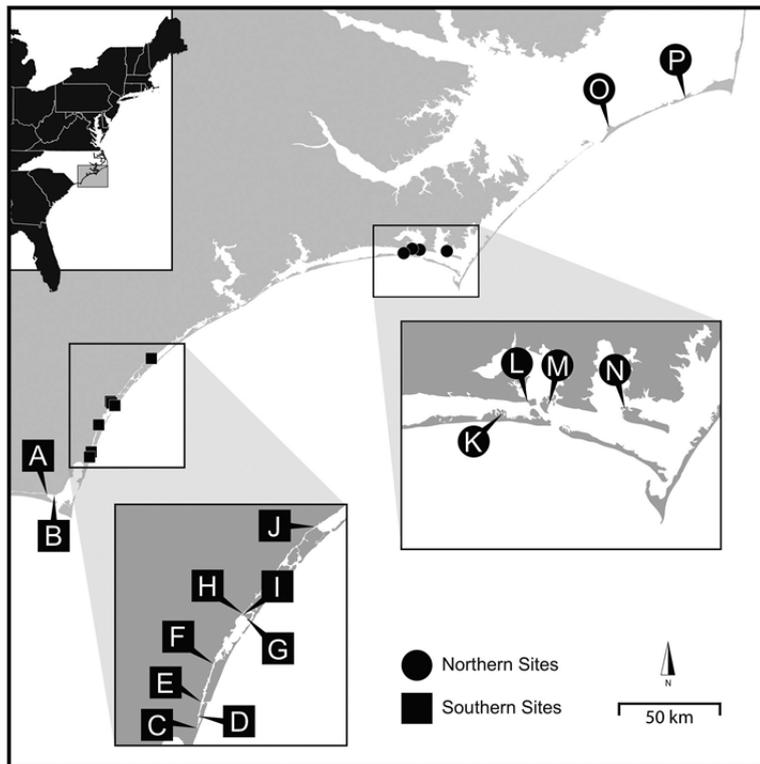


Table 1. Collection sites sampled in North Carolina and number of ascidian species observed at each site.

Code	Harbor/Marina Name	Latitude	Number of Ascidian Species		
			Native	Cryptogenic	Introduced
A	South Harbor Village Marina	South	1	0	1
B	Southport Marina	South	0	0	0
C	Harbour Point Marina	South	0	0	0
D	Joyner Marina	South	0	0	0
E	Inlet Watch Yacht Club	South	0	0	0
F	Masonboro Yacht Club & Marina	South	1	0	1
G	Seapath Yacht Club	South	4	2	3
H	Bridge Tender Marina	South	2	1	3
I	Wrightsville Beach Marina	South	3	0	3
J	Harbour Village Marina	South	1	0	2
K	Crow’s Nest Yacht Club	North	2	0	1
L	Portside Marina	North	3	0	2
M	Olde Towne Yacht Club	North	5	0	2
N	Harkers Island	North	1	0	1
O	Ocracoke Ferry Landing	North	1	0	1
P	Hatteras Harbor Marina	North	1	0	1

analysis; the remainder was placed in buffered 10% seawater formalin for taxonomic identification. For solitary species, one full individual was preserved in formalin, while a piece of the branchial sac of a second individual was dissected and fixed in 100% ethanol for DNA analysis. All ethanol-preserved samples were stored in a -20 °C freezer until DNA extraction. Specimen identification was achieved

using appropriate morphological keys and species’ descriptions (Van Name 1945; F Monniot 1974, 1983; C Monniot 1983a, b; Monniot and Monniot 1984; Goodbody 1994; Rocha et al. 2012b). Once identified, each species was classified as native, introduced, or cryptogenic according to Carlton (1996) and Blackburn et al. (2011). The term “introduced” refers to species well established in a non-native area

Table 2. Ascidian species observed and relative abundance at each site in July 2014. *A. st* = *Aplidium stellatum*, *A. in* = *Ascidia interrupta*, *C. ob* = *Clavelina oblonga*, *D. lu* = *Didemnum lutarium*, *D. be* = *Distaplia bermudensis*, *D. co* = *Distaplia corolla*, *D. st* = *Distaplia stylifera*, *E. ca* = *Eudistoma capsulatum*, *M. ma* = *Molgula manhattensis*, *P. vi* = *Perophora viridis*, *P. an* = *Polyandrocarpa anguinea*, *P. zo* = *Polyandrocarpa zorritensis*, *S. pl* = *Styela plicata*. 1 = present but rare (one or a few specimens of a species observed), 2 = common (species frequently observed but not overly abundant), 3 = abundant (species occurring frequently), 4 = very abundant (species occurring frequently and in great numbers or clusters). Codes correspond to map shown in Figure 1. Harbour Point Marina, Joyner Marina, Inlet Watch Yacht Club, and Southport Marina are not listed, because no ascidians were observed at these sites.

Code	Site	Ascidian species and relative abundance												
		<i>A. st</i>	<i>A. in</i>	<i>C. ob</i>	<i>D. lu</i>	<i>D. be</i>	<i>D. co</i>	<i>D. st</i>	<i>E. ca</i>	<i>M. ma</i>	<i>P. vi</i>	<i>P. an</i>	<i>P. zo</i>	<i>S. pl</i>
A	South Harbor Village Marina									2				1
F	Masonboro Yacht Club & Marina									1				3
G	Seapath Yacht Club	1	1		1	3		1	1			1	1	4
H	Bridge Tender Marina				1	1					1	1	4	4
I	Wrightsville Beach Marina		3							1	1	1	4	4
J	Harbour Village Marina		1									1	4	4
K	Crow's Nest Yacht Club		3								1			4
L	Portside Marina	1	2						1			1		4
M	Olde Towne Yacht Club	1	1	1			1		1			1		4
N	Harkers Island										1			1
O	Ocracoke Ferry Landing										1			1
P	Hatteras Harbor Marina										1			1

of reduced dimension (often a harbor or marina), where they are able to survive and reproduce. The term “cryptogenic” applies to these species that cannot reliably be classified as being either native or introduced (Carlton 1996). The status of each ascidian species was determined based on Shenkar and Swalla (2011), Zhan et al. (2015), Simkanin et al. (2016), and the “Ascidiacea World Database” (Shenkar et al. 2017).

Ascidian barcoding

One to four specimens per species were collected for barcoding purposes (Table 3). Zooids of colonial species were carefully dissected from the tunic. For solitary ascidians, a piece of the previously removed branchial sac was sub-sampled. Ethanol was fully evaporated using an Eppendorf® Vacufuge® centrifuge prior to DNA extraction using the DNeasy® Blood and Tissue Kit (QIAGEN) following manufacturer’s instructions. PCR amplification of a fragment of the mitochondrial gene COI was achieved using the universal primers LCO1490 and HCO2198 (Folmer et al. 1994) and the ascidian-specific primers Tun_forward and Tun_reverse2 (Stefaniak et al. 2009). PCR reactions for each sample consisted of 0.5 µL of each primer (10 µM), 11 µL of PCR water, 12.5 µL of MyTaq HS MIX (2X), and 0.5 µL of DNA for a total volume of 25 µL. PCR amplification cycles with the LCO1490 and HCO2198 primer set were as follows: 95 °C for 1 min, 35 amplification cycles (95 °C for 15 sec, 45 °C for 15 sec, and 72 °C for 10 sec), and a final extension step at

Table 3. Introduction status of ascidians identified in North Carolina (classification as native, introduced, or cryptogenic, Carlton 1996). Different GenBank® accession numbers for a given species correspond to different haplotypes.

Species	Origin	GenBank® Accession Numbers
<i>Aplidium stellatum</i>	Native	KY111411-14
<i>Ascidia interrupta</i>	Native	KY111415-16
<i>Clavelina oblonga</i>	Native	KY111417
<i>Didemnum lutarium</i>	Native	KY111418
<i>Distaplia bermudensis</i>	Cryptogenic	KY111419
<i>Distaplia corolla</i>	Native	–
<i>Distaplia stylifera</i>	Cryptogenic	–
<i>Eudistoma capsulatum</i>	Native	KY111420-23
<i>Molgula manhattensis</i>	Native	KY111424
<i>Perophora viridis</i>	Native	KY111425
<i>Polyandrocarpa anguinea</i>	Introduced	KY111426-28
<i>Polyandrocarpa zorritensis</i>	Introduced	KY111429
<i>Styela plicata</i>	Introduced	KY111430-32

72 °C for 1 min. For the Tun_forward and Tun_reverse2 primer set, conditions were as above but 40 cycles were conducted with an annealing temperature of 42 °C. PCR amplifications were obtained on an Eppendorf Mastercycler nexus X2. Samples were sequenced using BigDye™ terminator v.3.1 and the same primers used in the amplification step on an Applied Biosystems 3130xl genetic analyzer available at the UNCW Center for Marine Science. The resulting DNA sequences were aligned using the software Geneious (v. R8, Biomatters, Auckland, New Zealand) and compared with ascidian reference sequences available in GenBank® via BLASTn searches.

Data analysis

To compare ascidian diversity and structure across NC harbors and marinas, two similarity matrices were created. The first was based on presence-absence. The second was based on the relative abundance of each species found at each marina. No transformation of data was applied since our data were semi-quantitative. Matrices were constructed using the Bray-Curtis index and results were visualized with non-metric multidimensional scaling (nMDS) plots. Permutational analyses of variance (PERMANOVA) were applied to assess the effect of latitudinal position of the investigated harbors and marinas (South *versus* North, Figure 1) on ascidian diversity and distribution. Analyses were done using the Primer v6.1.10 statistical package (Clarke and Gorley 2006) with the PERMANOVA + β 20 module (Anderson et al. 2008) incorporated. Additionally, mantel tests were conducted to test for correlations between geographic distances among marinas and ascidian community dissimilarity for both the presence-absence and relative abundance data. GPS coordinates of the twelve sites were used to calculate the shortest surface distances between pairs of harbors using Byers (1997) software. Mantel tests were performed using the ade4 package for R and its significance tested by permutation (Dray and Dufour 2007).

Population dynamics over time

Data collection

Twelve transects were established on nine pilings supporting the Wrightsville Beach drawbridge located next to Bridge Tender Marina, Wilmington, NC (Figure 1 point H). Transects were established and visited at low tide once a month from July 2014 to November 2015. At each transect, 5 photos were taken (each 0.3 m apart vertically) from approximately 0 m (at the lowest tide) to 1.5 m depth by snorkeling with an Olympus C-7070 camera equipped with an underwater PT-027 housing fixed to an aluminum frame (outside edge: 13.208 cm \times 19.812 cm, inside edge: 11.716 cm \times 17.526 cm, length from lens to frame edge: 13 cm) to keep a constant focal distance. Salinity (measured with a refractometer) and water temperature measurements were also taken at the time of sampling as metadata (Table S2).

Image analysis

For each photograph, ascidian species were identified and abundance in numbers of individuals (solitary species) or colonies (colonial species) was recorded. We also measured the surface area

(percent cover) of each colonial species using ImageJ 1.48v software. The total areas for each individual species were divided by the total area of the photograph to obtain a percent cover per species for each photo. Newly recruited ascidians (i.e. individuals or colonies $< 0.5 \text{ cm}^2$) could not be positively identified and therefore were not quantified.

Data analysis

Abundance per cm^2 of all ascidian species and percent cover of colonial species were plotted against measured temperature values (Table S1) using Microsoft Excel[®] for Mac version 15.20. Cross-correlation analyses between temperature and species' percent cover or abundance over time (months) were conducted using the Pearson correlation coefficient and the software SYSTAT v.12.02. Correlation coefficient values at lag 0 are equivalent to the standard Pearson correlation (i.e. species' percent cover or abundance correlated with temperature values for that same month). Negative lags correspond to correlations between values of the first data series (species' percent cover or abundance) and values of the second series (temperature) 'n' months prior (negative lags) or later (positive lags).

Results

Ascidian diversity and distribution patterns

Four of the sixteen North Carolina harbors and marinas visited contained no ascidians: Harbour Point Marina, Joyner Marina, Inlet Watch Yacht Club, and Southport Marina; all of these sites were in the southern part of the sampling range (Table 1). Thirteen ascidian species were identified (Table 2) at the twelve remaining marinas (Figure S1): eight were native [*Aplidium stellatum* (Verrill, 1871); *Ascidia interrupta* Heller, 1878; *Clavelina oblonga* Herdman, 1880; *Didemnum lutarium* Van Name, 1910; *Distaplia corolla* Monniot F., 1974; *Eudistoma capsulatum* (Van Name, 1902); *Molgula manhattensis* (De Kay, 1843); and *Perophora viridis* Verrill, 1871]; two were cryptogenic [*Distaplia bermudensis* Van Name, 1902; and *Distaplia stylifera* (Kowalevsky, 1874)]; and three were introduced [*Polyandrocarpa anguinea* (Sluiter, 1898); *Polyandrocarpa zorritensis* (Van Name, 1931); and *Styela plicata* (Lesueur, 1823)]. The solitary species *Styela plicata* was the most widespread and abundant species, and was recorded at twelve sites.

At sites with ascidians, the number of species present ranged from 2 to 9 (Table 1). Some species were widespread along the NC coast (e.g., *Aplidium stellatum*, *Ascidia interrupta*, *Eudistoma capsulatum*,

Perophora viridis, *Polyandrocarpa anguinea* and *Styela plicata*), while others were exclusively recorded at southern sites (e.g. *Didemnum lutarium*, *Distaplia bermudensis*, *Distaplia stylifera*, *Molgula manhattensis* and *Polyandrocarpa zorritensis*) or northern sites (*Clavelina oblonga* and *Distaplia corolla*) (Table 2). However, the nMDS plots representing both presence-absence and relative abundance data showed no clear geographic pattern at the community level (Figure 2), an observation further supported by a lack of significant differences revealed with a PERMANOVA analysis ($p = 0.158$ and $p = 0.159$, respectively). Similarly, a mantel test considering geographic distance and ascidian community similarity showed no significant correlation for presence-absence data ($p = 0.106$). However, the relative number of individuals per species present at each harbor was significantly related to the distance between sites ($p = 0.019$).

All ascidians were successfully barcoded (GenBank® accession numbers KY111411 to KY111432, Table 3), except for *Distaplia corolla* and *Distaplia stylifera* for which no amplification was obtained possibly due to a mutation in the primer annealing site.

Population dynamics over time

Five ascidian species were identified at the photo transect site established at Wilmington, NC: the colonial ascidians *Didemnum lutarium*, *Distaplia bermudensis*, *Eudistoma capsulatum*, and the solitary ascidians *Ascidia interrupta* and *Styela plicata*. Seasonal patterns in abundance or percent cover were observed for all species (Figures 3, 4, S2). In terms of abundance (Figure 3A), both *Didemnum lutarium* and *Distaplia bermudensis* colony numbers increased in the fall and winter and persisted until late spring, when their numbers began to decrease. Accordingly, cross-correlation analyses for both species showed a significant negative correlation (Figure 4B and 4C respectively), indicating that an increase in total number of colonies for these species was concomitant with a temperature decrease (that same month for *Didemnum lutarium* and during the same month and 1 and 2 months prior for *Distaplia bermudensis*). Though there did not seem to be a strong influence of temperature on the seasonal abundance of *Styela plicata*, there was a significant negative correlation between *Styela plicata* abundance and temperature during the two months prior (Figure 4E). An important decline in the total number of individuals of *Styela plicata* was observed in summer, after the maximum temperature for the year was reached (June 2015: 29.3 °C, Table S2). The reverse pattern was observed for the solitary species

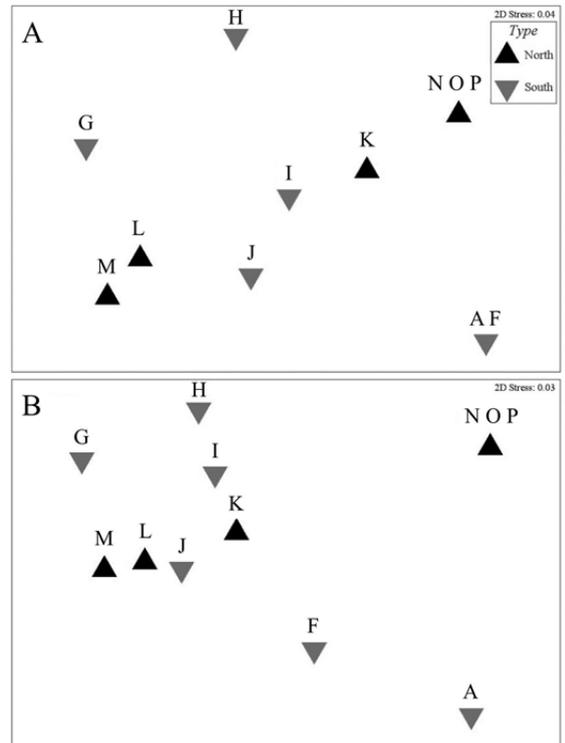


Figure 2. Non-metric multidimensional scaling (nMDS) plots of latitudinal position (South = grey, North = black) versus presence-absence (A) and relative abundance (B) of ascidian species. Letters represent harbors listed in Table 1 and 2, and Figure 1.

Ascidia interrupta, which exhibited increase abundance in summer (Figure 3A) and a significant positive correlation with temperatures (time lag 0; Figure 4A). Absolute number of colonies for *Eudistoma capsulatum* did not appear to be influenced by seasonal water temperature changes, and there was no significant correlation between abundance and temperature (Figure 3A and 4D).

The percent cover of *Didemnum lutarium* and *Distaplia bermudensis* varied in accordance with changes in their abundances, with both species exhibiting higher percent cover in winter (Figure 3B). Accordingly, percent cover was negatively correlated with temperature values for *Didemnum lutarium* (time lag 0 to -1 respectively) and *Distaplia bermudensis* (Figure S2A and S2B, respectively). In terms of coverage, *Eudistoma capsulatum* was the most abundant species and was present at high abundances (an average of 0.0062 colonies/cm² ± 0.01, SD) all year long (Figure 3A). Although no significant cross-correlation was observed between seawater temperature and abundance of *Eudistoma capsulatum* colonies (Figure 4D), percent cover appeared to be slightly higher in late summer and fall (Figure 3B).

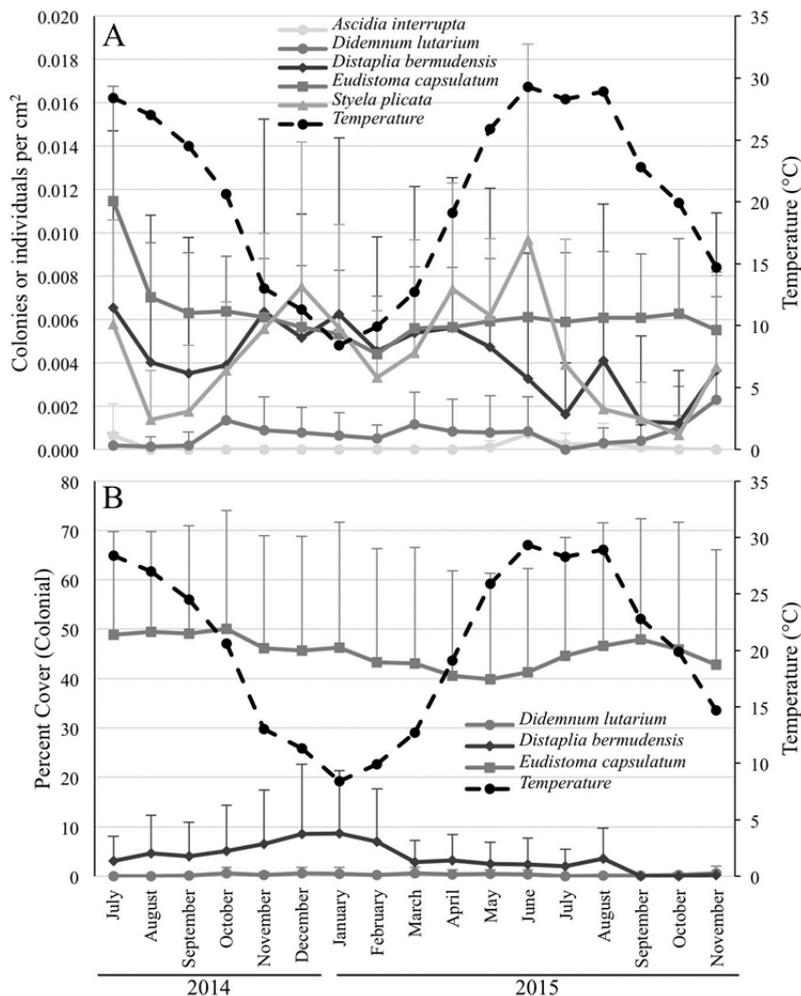


Figure 3. Abundance (number of colonies or individuals, \pm SD) of five ascidian species NC (A); percent cover (\pm SD) of three colonial ascidian species (B) in photo transects at Wilmington, NC from July 2014 to November 2015.

Accordingly, a significant positive correlation was found for the percent coverage of this species and seawater temperatures. In particular, an increase in percent coverage was preceded by a temperature maximum 3–4 months beforehand (time lag -3 and -4, Figure S2C).

Discussion

This study documented the occurrence of thirteen ascidian species in 12 harbors and marinas along the North Carolina coast. Eight of these species were considered native, two cryptogenic, and three introduced. The globally distributed solitary ascidian *Styela plicata* (Barros et al. 2009; Pineda et al. 2011) was the most widespread, and often the most abundant, ascidian species in NC harbors and marinas. The latitudinal location of each harbor did not appear to have an effect on community similarity

in either the presence-absence or the relative abundance of ascidians. Similarly, the distance between harbors was not associated with differences in the presence-absence of ascidian species. There were species found only at southern and northern sites (five species in only southern sites, and two in only northern sites), but their presence was too sporadic to be biologically meaningful. Geographic distances between harbors did have an effect on the community composition in terms of the relative abundance of ascidian species. These results suggest that some environmental conditions such as seawater temperature, low salinity episodes, or current patterns among distantly located harbors are distinct enough to foster success of different species.

The most abundant species at the Wilmington site were the native species *Eudistoma capsulatum*, the cryptogenic *Distaplia bermudensis* and the introduced *Styela plicata*. These three species were

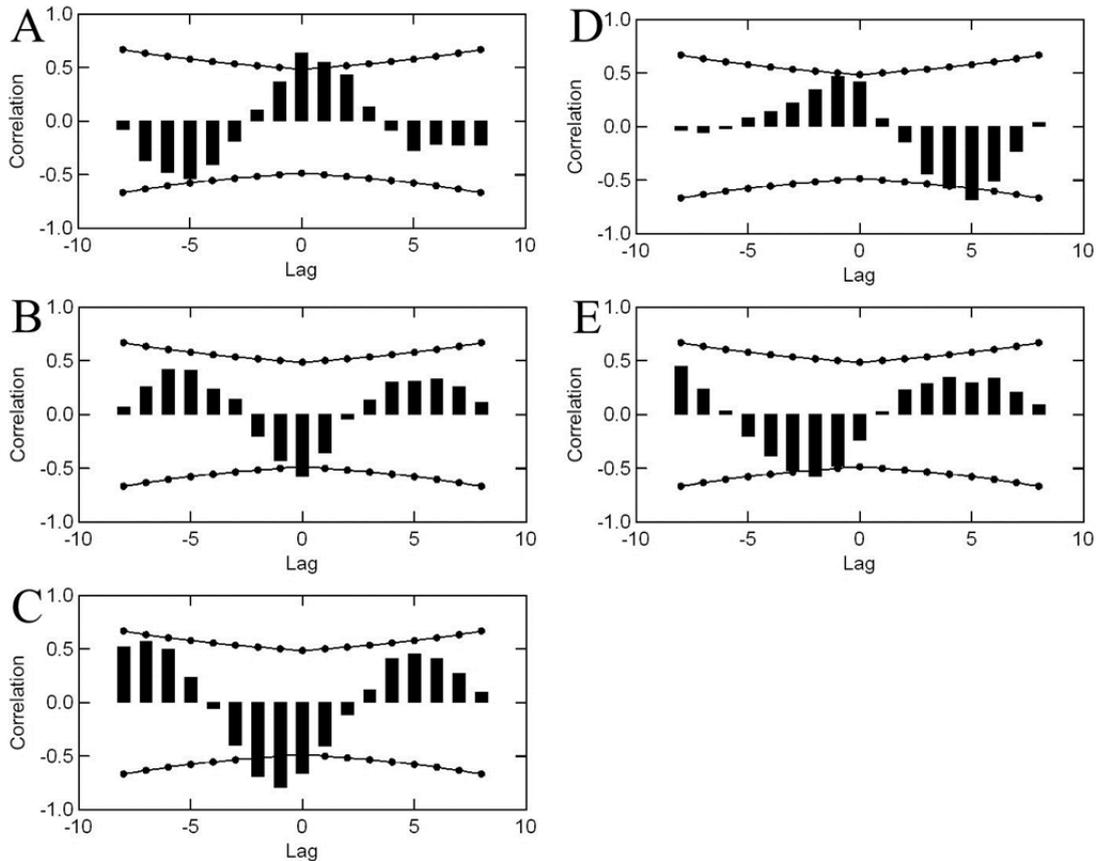


Figure 4. Cross-correlation of water temperature in relation to temporal abundance for *Ascidia interrupta* (A), *Didemnum lutarium* (B), *Distaplia bermudensis* (C), *Eudistoma capsulatum* (D), and *Styela plicata* (E) at Wilmington, NC. Time lag is in months. Dotted lines along top and bottom represent significance (bar crossing line = significant).

repeatedly observed competing for space, with the colonial ascidians *Eudistoma capsulatum* and *Distaplia bermudensis* often growing over *Styela plicata* (Figure S3). Abundance and colony coverage of *Eudistoma capsulatum* were relatively stable, while abundances of *Distaplia bermudensis* and *Styela plicata* varied over time. A clear seasonal pattern was found for the five species studied over a year and a half: adult colonies of *Didemnum lutarium*, *Distaplia bermudensis* and individuals of *Styela plicata* were more abundant during the colder months of the year, while *Ascidia interrupta* preferred the warmer months and *Eudistoma capsulatum* colonies appeared to thrive all year long with a peak in late summer and fall. Other temperate ascidians are known to show seasonal patterns correlated with temporal changes in seawater temperature (Turón and Becerro 1992; Ribes et al. 1998; López-Legentil et al. 2005, 2013; Dijkstra et al. 2007). The pattern recorded here for the introduced species *Styela plicata*, the cryptogenic *Distaplia bermudensis* and

the native *Didemnum lutarium* were similar to what has been found for temperate ascidians in the northwestern Mediterranean Sea within the order Aplousobranchia, to which *Distaplia bermudensis* and *Didemnum lutarium* belong. The polycitorid *Cystodytes dellechiaiei* (Della Valle, 1877), the polyclinids *Pseudodistoma crucigaster* Gaill, 1972 and *Aplidium* aff. *conicum* (Olivi, 1792), and the didemnids *Polysyncrator lacazei* (Giard, 1872), *Diplosoma spongiforme* (Giard, 1872) and *Didemnum fulgens* (Milne Edwards, 1841) have been shown to have reduced growth during the summer when temperatures are highest (Coma et al. 2000), and active growth during the winter (Turón and Becerro 1992; López-Legentil et al. 2005, 2013). Contrary to what we observed for the solitary ascidian *Ascidia interrupta*, no temperate ascidian species were observed to actively increase in numbers during the summer months in the Mediterranean Sea, even though maximum seawater temperatures (≤ 27.5 °C; Garrabou et al. 2009) are lower than those recorded

in this study (Table 1). *Styela plicata* has been previously documented to suffer significant mortality around June in NC because of extreme physiological stress related to sharp increases in temperature (Pineda et al. 2012b). Here we observed a similar pattern, with high abundances of *Styela plicata* from early fall through early spring, and low abundances during the summer.

The three COI sequences obtained in this study for *Styela plicata* corresponded to haplotypes 1, 2 and 14 from Pineda et al. (2016). Haplotypes 1 and 2 were the most common found at UNCW Center for Marine Science (approximately 1 mile south of Masonboro Yacht Club & Marina) from 2007 to 2009, while haplotype 14 was only recorded in 2007 and 2009 (Pineda et al. 2016). All haplotypes found here were classified within Group 2 (Pineda et al. 2011). Group 2 is the smaller (in number of haplotypes) of the two observed groups worldwide and is formed by 8 haplotypes, 6 of which were exclusively found in NC (private haplotypes; Pineda et al. 2011). Similarly, *Polyandrocarpa zorrissentis* is a widely distributed species of uncertain origin (Sanamyan and Monniot 2007a) but first described from Peru (Van Name 1931). This species is widespread along the Pacific, Atlantic and Gulf coasts of the U.S.A. (Lambert and Lambert 1998, 2003; Simkanin et al. 2016); however, to date no COI sequence was available for specimens collected in the U.S.A. In fact, the best BLASTn match (99% identity, 100% coverage) for the haplotype obtained in this study was with a sequence from a specimen collected in northeastern Spain (López-Legentil et al. 2015). *Polyandrocarpa anguinea* is reported here for the first time in the U.S.A. This species was described by Sluiter (1898) in Knysna, South Africa and has since been reported in Martinique, Brazil, Panama, Sierra Leone, Mauritius Island, Indonesia, Philippines, Australia, New Caledonia and the Mediterranean Sea (Brunetti and Mastrototaro 2004; reviewed in Carman et al. 2011). Until this study, no barcoding sequence for this species was available in GenBank®.

Two other species have global distributions but are considered native to the North American Atlantic coast, *Clavelina oblonga* and *Perophora viridis*. *Perophora viridis* was recorded in six of the harbors visited, in three of these it co-occurred with *Styela plicata*. *Perophora viridis* has also been observed in the Mediterranean and Caribbean Seas (Sanamyan and Monniot 2007b). Although this species is widely distributed, only a sequence for a specimen collected in South Carolina (U.S.A.) was available in GenBank® and presented 98% identity (100% coverage; Stach and Turbeville 2002) with the sequence obtained here. *Clavelina oblonga* was first described in Bermuda

(Herdman 1880) and is considered native to the Caribbean Sea and southern Atlantic coast of North America; it has been introduced to the Azores, Brazil, Cape Verde, Senegal, and Spain (Rocha et al. 2012a; López-Legentil et al. 2015; Ordoñez et al. 2016). In North Carolina, we observed this species only at the Olde Towne Yacht Club in Beaufort. The barcoding sequence obtained in this study was 99% identical to several sequences published in GenBank®, including samples from Brazil (Rocha et al. 2012a), Spain (López-Legentil et al. 2015) and the Azores (Turon and López-Legentil 2004). Even though the species is considered native to the southern U.S. Atlantic coast, the ancestors of the colonies of *Clavelina oblonga* observed at Beaufort could have arrived attached to a ship hull. Thus, it is unclear whether the genotype obtained here for *Clavelina oblonga* can be considered a native genotype for this species in NC.

High reproductive capacity, rapid population growth rates, and wide degrees of tolerance to environmental fluctuations are some of the factors that allow introduced species to prevail over native ones (Lambert 2005). Introduced species have been shown to persist and be more resilient to rapidly warming seawater temperatures than native species (Yamaguchi 1975; Sutherland 1978; Rocha 1991; Vermeij 1996; Stachowicz et al. 2002). Accordingly, in many harbors and marinas worldwide, introduced ascidians are more prevalent than native ones (Lambert and Lambert 1998, 2003; Marins et al. 2010; Tracy and Reynolds 2014; López-Legentil et al. 2015). However, more native than introduced ascidian species were observed in NC harbors and marinas. This suggests that habitat disruption by artificial structures, pollution related to recreational and commercial boating in the zone, and the presence of a few introduced species have not completely altered the conditions required for native species to thrive in the studied area. Some factors that may contribute to the prevalence of native versus introduced species in NC include low pollution levels in NC harbors and exposure to strong currents that may facilitate the arrival of larvae from nearby habitats. Alternatively, it is possible that some species considered native were in fact introduced before records were kept for the area. Clearly, it is paramount to perform regular and thorough inventories of species inhabiting our coasts to detect the arrival of new species and to monitor the spread of established ones. Moreover, since introduced species can often outcompete native species, knowing more about their life cycles and environmental thresholds may help elucidate possible patterns of spread, the likelihood of a species to become invasive, and allow for the design of appropriate eradication or contingency plans.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Collection sites sampled in North Carolina, USA.

Table S2. Sampling dates of photo transects at Bridge Tender Marina with water temperature and salinity values measured at the time of sampling.

Figure S1. Ascidian species collected in NC harbors and marinas.

Figure S2. Cross-correlation plots of temperature vs. percent cover of colonial species over time.

Figure S3. The three most common ascidian species at Wilmington, NC.

This material is available as part of online article from:

http://www.aquaticinvasions.net/2017/Supplements/AI_2017_Villalobos_et_al_Supplementary_tables.xls

http://www.aquaticinvasions.net/2017/Supplements/AI_2017_Villalobos_et_al_Supplementary_figures.pdf