

## Diversity and abundance of native and non-native ascidians in Puerto Rican harbors and marinas

Olivia T. Streit<sup>a</sup>, Gretchen Lambert<sup>b</sup>, Patrick M. Erwin<sup>a</sup>, Susanna López-Legentil<sup>a,\*</sup>

<sup>a</sup> Department of Biology & Marine Biology, Center for Marine Science, University of North Carolina Wilmington, Wilmington, NC 28403, USA

<sup>b</sup> University of Washington Friday Harbor Laboratories, Friday Harbor, WA 98250, USA

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### ABSTRACT

Ascidians are an ideal taxon to study invasion processes: they require anthropogenic introduction vectors for long-distance dispersal, are easy to collect and monitor, and are abundant on artificial substrates. In March 2019 we surveyed 11 harbors around Puerto Rico and recorded 47 ascidian species. Eleven of these were only identified to the genus level or above based on morphological or genetic characterization. The remaining 36 species were classified as: 11 introduced (7 with worldwide distributions), 13 cryptogenic, and 12 native. We report the occurrence of *Phallusia* cf. *philippinensis* in the Atlantic for the first time. Ascidian community structure did not differ significantly across geographic locations and distances between marinas, while marina size had a significant effect on species richness and composition. Stakeholder involvement and periodic monitoring efforts are essential to detect the arrival of new species and the spread of already introduced ones to natural habitats.

### 1. Introduction

Anthropogenic transport has expedited the invasion process for many marine species, resulting in increased numbers of introduced non-native species in many regions. In San Francisco Bay, for example, the rate of introductions has increased from one species every 55 weeks to one species every 14 weeks in just over 30 years (Cohen and Carlton, 1998). International shipping facilitates the transportation of thousands of species around the world (Carlton and Geller, 1993; Paulay et al., 2002; Godwin, 2003). Most introduced species remain limited to artificial substrates with a few able to colonize natural benthic substrates and affect ecosystem functioning (Ruiz et al., 1999; Lambert, 2002; Simberloff, 2011). Species like the ascidian *Didemnum vexillum* have established huge subtidal populations and completely changed native communities (Lambert, 2009). Recreational boating among harbors further increases dispersal of species attached to hulls, increasing propagule pressure over time and the probability of non-native species establishing viable populations (Wasson et al., 2001; López-Legentil et al., 2015; Zhan et al., 2015; Simkanin, 2016). Accordingly, highly frequented harbors are at higher risk of invasion (Darbyson, 2009; Seebens et al., 2013), a risk that increases with length of time dockside (Darbyson, 2009). In some areas, recreational boat movement is such that it has resulted in the introduction of more non-native species than

has commercial ship traffic (e.g., southern Gulf of St. Lawrence; Darbyson, 2009).

Ascidians (Phylum Chordata) are commonly found in marinas worldwide and represent ideal models to study the importance of anthropogenic transport for species introduction (Zhan et al., 2015). Ascidians are the largest and most diverse class within the subphylum Tunicata, with over 3000 species documented (Shenkar and Swalla, 2011). This taxon also has one of the highest numbers of marine introduced species, with an estimated 80 species documented outside of their native range (Zhan et al., 2015). Ascidians are sessile as adults and have short-lived lecithotrophic larvae usually viable for around a day (Svane and Young, 1989; Lambert, 2005). Thus, natural dispersal is very limited and reduced to rafting or drifting of gametes and embryos or fragments of colonial species (Davis and Butler, 1989; Ayre et al., 1997). More frequently, long distance transport of ascidians is achieved through anthropogenic vectors (e.g., attached to boat hulls, sea chests or aquaculture cages; Bullard et al., 2007; Coutts and Dodgshun, 2007; Aldred and Clare, 2014; Zhan et al., 2015).

Several life-history attributes have contributed to the rapid establishment of some ascidian species worldwide: rapid growth, high rates of reproduction, adaptability to variable environmental factors such as temperature and salinity, and long reproductive lifespans (Lambert, 2005; Pineda et al., 2012a, 2012b, 2013; Rocha et al., 2017). All

\* Corresponding author.

E-mail address: [lopezlegentils@uncw.edu](mailto:lopezlegentils@uncw.edu) (S. López-Legentil).

ascidians are hermaphrodites; some species are capable of producing larvae throughout the year (Kremer et al., 2010; Pineda et al., 2013), while others are capable of self-fertilization (Morgan, 1942; Lambert, 2004; Jiang and Smith, 2005; Phillippi and Yund, 2017). In addition, all colonial and some solitary species are brooders (Lambert, 2004, 2005). These traits allow for rapid colonization of a new habitat by only a few individuals, often outcompeting native species for resources such as space (Rius et al., 2009; Kremer et al., 2010; Pineda et al., 2013). The combination of these attributes and sustained ship traffic between marinas has led to non-native ascidians being more abundant than native species in many harbors (López-Legentil et al., 2015).

In Puerto Rico, the first ascidian survey was conducted 90 years ago. Van Name (1930) documented 26 species for the island, and later reported an additional one, *Ecteinascidia conklini* (Van Name, 1945). Most of the species reported in Van Name (1930) were collected by either dredging or collecting from intertidal natural benthic surfaces, with additional samples taken from wharf pilings in Guanica Bay. Van Name (1930) stated that most of the species had a wide distribution and that “The species thus far known from Porto Rico are ... only a little more than 53 per cent of those recorded from all the West Indies. No doubt this percentage will be greatly increased by future collecting”. Among Van Name’s records were six species now considered introduced to the Caribbean Sea: *Diplosoma listerianum*, *Aplidium lobatum*, *Ascidia sydneiensis*, *Rhodosoma turcicum*, *Styela canopus*, and *S. plicata*, and a number of others now considered cryptogenic. Toffart (1983) focused on identifying benthic macroorganisms attached to mangrove roots in Guadeloupe. In his work, Toffart (1983) also reported 27 ascidian species in Puerto Rican mangroves, two of which were only identified to the genus level (a *Didemnum* sp. and a *Botrylloides* sp.) and thus were not included in Table S1. To date, these are the only two studies on Puerto Rican ascidians. To investigate current ascidian communities, we visited 11 harbors and marinas around the Puerto Rican coast and identified all ascidians present using morphological and genetic tools, and compared our records with those of Toffart (1983) and Van Name (1930, 1945). Species were classified as native, cryptogenic or introduced and their

relative abundance and distribution determined to identify areas at higher risk of invasion. We hypothesized that geographic location and harbor size would influence the diversity and composition of native and introduced species in ascidian communities. We expected to find greater species diversity and distinct communities in marinas on the northern (more populated) coast compared to the southern coast, and in larger marinas compared to smaller marinas.

## 2. Materials and methods

### 2.1. Sample collection and processing

Surveys were conducted at 11 harbors and marinas around the Puerto Rican coast during March 8–13, 2019 (Fig. 1, Table 1). Water temperature and salinity were also recorded for each sampling location (Table 1). Surveys utilized a variation of Lambert and Lambert (1998) and the Rapid Assessment Method (Campbell et al., 2007), with relative abundance estimated immediately after the site survey was completed: 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 in great numbers or clusters of individuals. Specimens for morphological and genetic identification were collected from 0 to 2 m below the surface from artificial substrates including floating docks, boat bumpers and submerged ropes. All samples collected in Club Deportivo del Oeste, Marina Pescaderia and Club Náutico La Parguera were obtained from numerous ropes since these harbors lacked floating docks. Whenever possible, specimens were photographed *in situ* prior to collection.

After collection, samples were placed in plastic trays filled with seawater and representative samples of each species were assigned a sample code, photographed, and processed as follows. For genetic identification, colonial species were preserved whole, while solitary species were dissected *in situ* to remove the tunic before fixing the body in 95% ethanol. Within a week, all ethanol samples were carefully rinsed

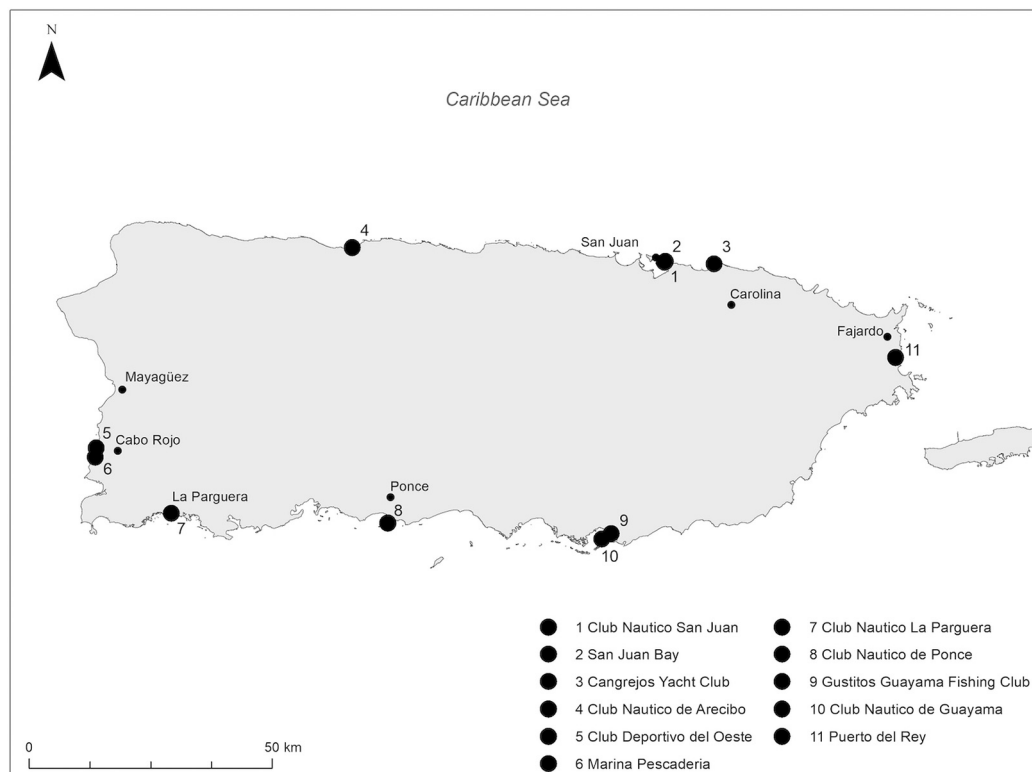


Fig. 1. Map indicating harbors and marinas surveyed in Puerto Rico. Northern sites (1–4, 11), southern sites (5–10).

**Table 1**

The 11 Puerto Rican marinas surveyed during this study with geographic location of the island (North or South), 2019 survey date, marina size (small: < 100 slips; medium: 100–199 slips; and large: > 200 slips), temperature (°C), and salinity (‰) at each site.

Marina	Region	Date	Size	Temperature	Salinity
Club Náutico San Juan	North	March 8	Medium	26.5	36
San Juan Bay Marina	North	March 8	Medium	26.5	36
Cangrejos Yacht Club	North	March 8	Large	28.5	36
Club Náutico Arecibo	North	March 9	Small	27.5	25
Club Deportivo del Oeste	South	March 10	Small	26.6	34
Marina Pescaderia	South	March 10	Small	27.6	36
Club Náutico La Parguera	South	March 11	Small	26.8	35
Ponce Yacht and Fishing Club	South	March 11	Medium	27	35
Gustitos Guayama Fishing Club	South	March 12	Small	26.3	34
Club Náutico de Guayama	South	March 12	Small	27.1	34
Puerto del Rey Marina	North	March 13	Large	26.2	33

with 95% ethanol and then stored in 100% ethanol in a – 20 °C freezer. For morphological analyses, specimens were relaxed for several hours in Ziploc® bags filled with seawater and a few drops of menthol dissolved in ethanol before preservation in 10% seawater formalin buffered with sodium borate. Taxonomic identification was conducted using appropriate morphological keys and species descriptions (Van Name, 1930, 1945; Berrill, 1932; Millar, 1962, 1974; Millar and Goodbody, 1974; Goodbody, 1984, 1993, 1995, 2000, 2003, 2004; Monniot, C., 1972a, b, 1983a, b, c; Monniot and Monniot, 1984; Monniot, F., 1972, 1983a, b, c; Rocha et al., 2005; Goodbody and Cole, 2006; Bonnet and Rocha, 2011). Once identified, each species was classified as native, introduced, or cryptogenic as described in Carlton (1996, 2009) and Blackburn et al. (2011). The term ‘introduced’ refers to species well established (i.e., able to survive and reproduce) in a non-native area of reduced dimensions (e.g., a harbor). The term ‘cryptogenic’ applies to species that cannot reliably be classified as either native or introduced (Carlton, 1996, 2009). Species were assigned to each status based on Shenkar and Swalla (2011), Zhan et al. (2015), and Simkanin (2016).

## 2.2. Ascidian barcoding

Whenever possible, at least one specimen per species was collected for genetic barcoding. To maximize DNA yield, zooids of colonial species and a piece of the branchial sac of solitary species were carefully dissected under a stereomicroscope prior to DNA extraction. Ethanol was evaporated using an Eppendorf® Vacufuge® centrifuge and DNA extracted using the DNeasy® Blood and Tissue Kit (QIAGEN) following manufacturer’s protocols. PCR amplification of a fragment of the mitochondrial gene Cytochrome Oxidase I (COI) was performed using the universal primers LCO1490 and HCO2198 (Folmer et al., 1994) or the ascidian-specific primers Tun\_forward and Tun\_reverse2 (Stefaniak et al., 2009). PCR reactions for each sample consisted of 1 µL of each primer (10 µM), 10.5 µL of PCR water, 12 µL of MyTaq HS MIX (2×), 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 with 95 °C for 15 s, 45 °C for 15 s, and 72 °C for 10 s, and a final extension step at 72 °C for 1 min. For the Tun\_forward and Tun\_reverse2 primer set, conditions were the same as described above except for the number of amplification cycles (40 instead of 35) and an annealing temperature of 42 °C instead of 45 °C. PCR

amplifications were conducted 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 3500 genetic analyzer available at UNCW Center for Marine Science. Resulting DNA sequences were aligned using the Geneious software (v. R11.1.5 Biomatters, Auckland, New Zealand) and deposited in GenBank® (accession numbers MT637936 to MT637989).

## 2.3. Data analysis

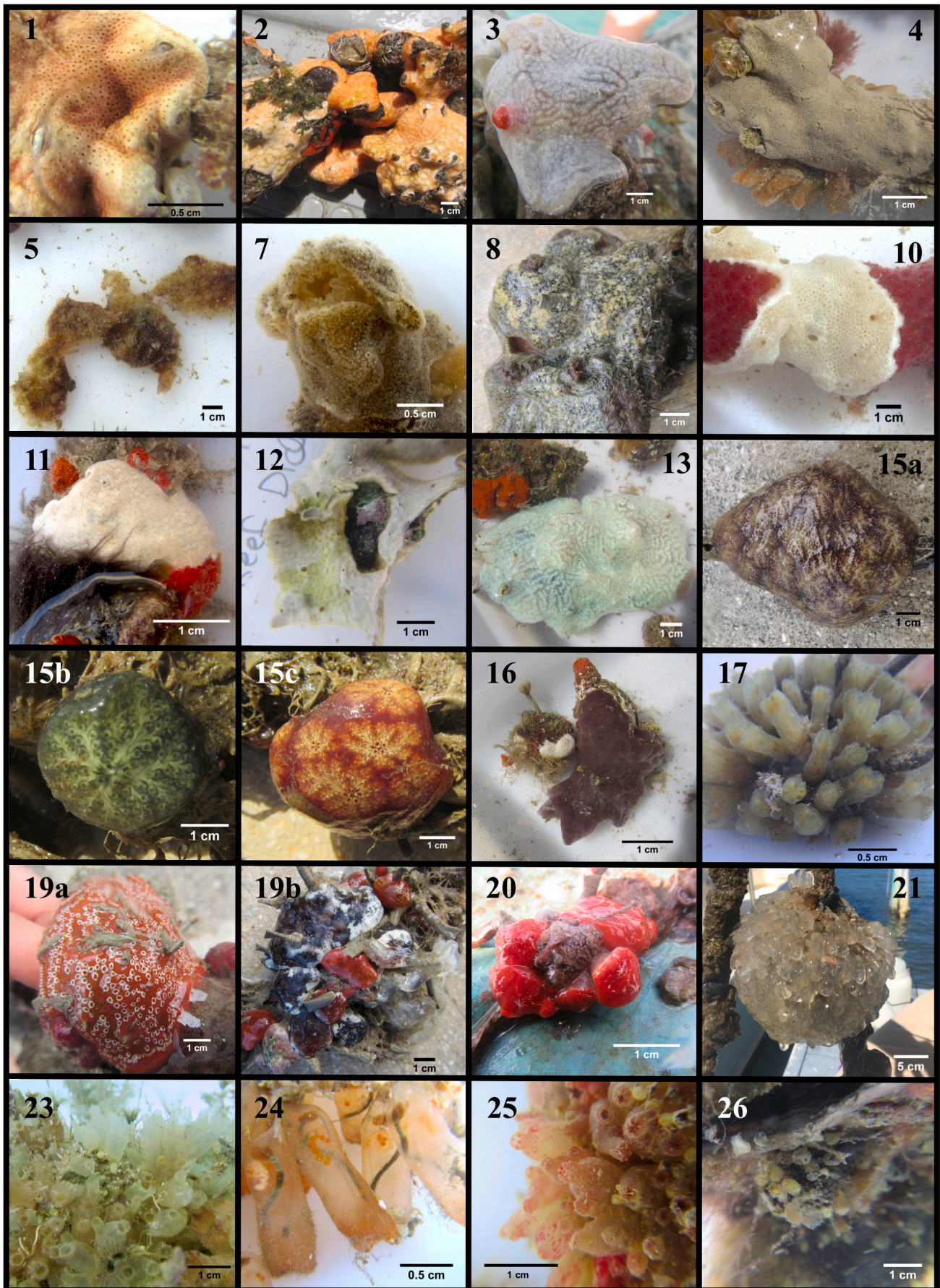
To compare ascidian diversity and structure across Puerto Rican harbors and marinas, univariate and multivariate statistical tests were conducted on the factors ‘geographic area’ (North vs. South) and ‘harbor size’ (small: < 100 slips; medium: 100–199 slips; and large: > 200 slips; Table 1). Note that five of the six small marinas are located in the South, and all the large marinas are in the North. Statistical analyses were conducted on the entire dataset (47 species) and two partitions corresponding to data from only ‘native species’ and only ‘introduced species’. For univariate analyses, parametric tests were conducted following non-significant ( $P > 0.05$ ) outcomes of normality (Shapiro-Wilk) and equal variance tests. Species richness was calculated for each site and compared across factors using a Student’s *t*-test for the factor ‘geographic area’ (2 factor levels) and analyses of variance (ANOVA) for ‘harbor size’ (3 factor levels) followed by Tukey’s honest significant difference (HSD) tests for multiple pairwise post hoc comparisons. For multivariate analyses, two similarity matrices were created using the Bray-Curtis index, one based on presence-absence data and the second based on relative abundance data for each species. Since the data was semi-quantitative, no transformation was applied. Results were visualized with nonmetric multidimensional scaling (nMDS) plots and compared across factors using permutational analyses of variance (PERMANOVA) in the PRIMER v6.1.10 statistical package (Clark and Gorley, 2006) with the PERMANOVA + Beta20 module (Anderson et al., 2008). Mantel tests were also conducted to assess correlations between geographic distances among marinas and ascidian community dissimilarity for both presence-absence and relative abundance matrices. GPS coordinates of the eleven 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 with significance testing by permutation (Dray and Dufour, 2007). To assess risk of invasion, a heat map depicting the percentage of introduced species relative to total species in each marina was created using ArcGIS (Lazaro et al., 2017).

## 3. Results

Only one of the 11 harbors visited (Club Náutico de Arecibo) had a salinity value that prevented ascidian establishment (Table 1); thus, no species were recorded and the marina was excluded from further analysis. A total of 47 ascidian species were observed in the remaining 10 Puerto Rican harbors surveyed based on morphology or unique mitochondrial sequences (Fig. 2; Table 2; Table S1). Species richness ranged from 11 in Club Deportivo del Oeste (south-western coast) and Gustitos Guayama Fishing Club (southern coast) to 25 species in Club Náutico de San Juan (northern coast). Of the 47 species, 11 were only identified to the genus level or above because specimens were rare and immature, and lacked discerning characters. The remaining 36 species were classified as: 11 introduced (7 with worldwide distributions, Fig. 3), 13 cryptogenic, and 12 native, and were distributed around the island as shown in Fig. 4 and Table S1. COI sequences were obtained for 34 of the species (Table 2). For the others, we either failed to obtain amplifications or we lacked tissue samples (e.g., *Microcosmus helleri*, *Styela* sp. 1).

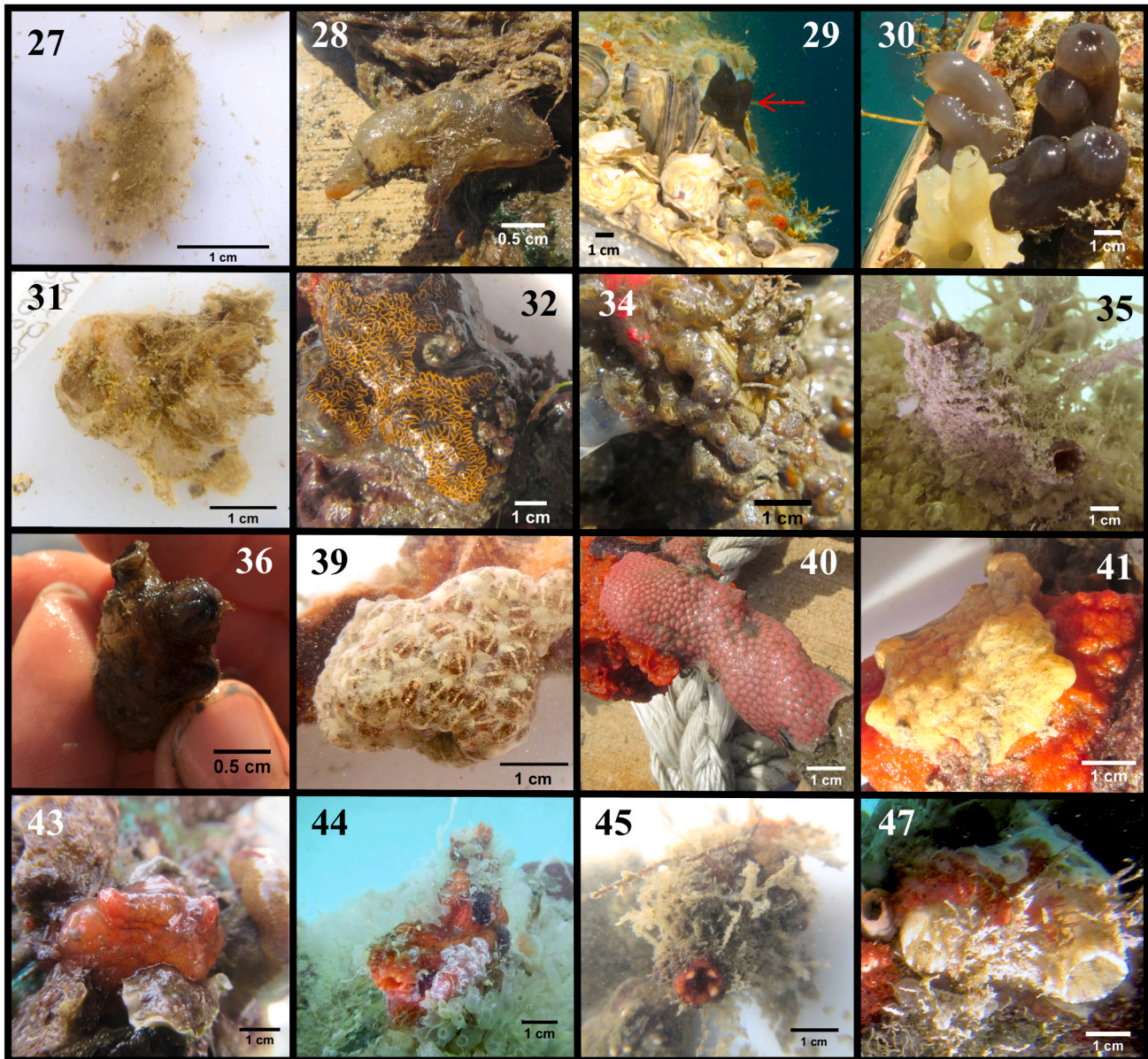
Marina size had a larger effect on the species richness of ascidian communities than geographic location (Fig. 5). Large and medium harbors consistently exhibited greater richness than small harbors, with a significant impact of harbor size on overall ascidian richness ( $F_{2,7} =$







**Fig. 2.** Ascidian species observed in Puerto Rican harbors and marinas. Aplousobranchia: 1) *Didemnum conchylatum*; 2) *D. duplicatum*; 3) *D. perlucidum*; 4) *D. psammotodes*; 5) *Didemnum* cf. *cineraceum*; 6) *Didemnum* sp. 1 – No picture; 7) *Diplosoma listerianum*; 8) *Diplosoma* sp. 1 (thick tunic); 9) *Diplosoma* sp. 2 – No picture; 10) *Lissoclinum fragile*; 11) Didemnidae 1; 12) *Trididemnum savignii*; 13) *Aplidium* cf. *antillense*; 14) *Aplidium* sp. 1 (clear tunic) – No picture; 15) *Polyclinum constellatum*, a. brown morph, b. green morph, c. red morph; 16) *Eudistoma* cf. *capsulatum*; 17) *E. olivaceum*; 18) *Eudistoma* sp. 1 – No picture; 19) *Distaplia bermudensis*, a. red with white siphons, b. different color morphs; 20) *D. stylifera*; 21) *Clavelina oblonga*; 22) Aplousobranchia 1 – No picture. Phlebobranchia: 23) *Ecteinascidia conklini*; 24) *E. turbinata*; 25) *Ecteinascidia* sp. 1; 26) *Perophora viridis*; 27) *Ascidia interrupta*; 28) *A. sydneyensis*; 29) *Phallusia nigra*; 30) *P.* cf. *philippinensis*; 31) *Rhodossoma turcicum*. Stolidobranchia: 32) *Botrylloides niger*; 33) *Botryllus primigenus* – No picture; 34) *Polyandrocarpa zorritensis*; 35) *Polycarpa spongiabilis*; 36) *Styela canopus*; 37) *Styela* sp. 1 – No picture; 38) *S. plicata* – No picture; 39) *Symplegma brakenhielmi*; 40) *S. rubra*; 41) *S. viride*; 42) *Symplegma* sp. 1 – No picture; 43) *Herdmania pallida*; 44) *Microcosmus exasperatus*; 45) *M. helleri*; 46) *Microcosmus* sp. 1 – No picture; 47) *Pyura vittata*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).



**Fig. 2.** (continued).

12.453,  $P = 0.005$ ) and native ascidian richness ( $F_{2,7} = 7.418$ ,  $P = 0.019$ ). Significant pairwise differences were detected between medium and small harbors when considering all ascidians, and between large and small harbors when considering only native ascidians (Fig. 5). Similar data trends were observed for introduced ascidian richness across marina size, though these differences were not significant ( $F_{2,7} = 3.480$ ,  $P = 0.089$ ). Marinas on the northern coast of the island were larger and had more species than marinas in the southern coast, with northern marinas having a mean of  $21.00 \pm 1.3$  (SE) and southern marinas  $15.33 \pm 1.8$  species; however, these differences were not statistically

significant ( $F_{1,8} = 4.999$ ,  $P = 0.056$ ; Fig. 5). Similarly, there were more native ascidian species in northern marinas compared to southern marinas (Fig. 5) but this difference was not significant ( $F_{1,8} = 3.136$ ,  $P = 0.115$ ). The trend of increased species richness in northern versus southern marinas was also detected for introduced species and was statistically significant ( $F_{1,8} = 6.154$ ,  $P = 0.038$ ). Six species were only recorded in northern marinas. Two of these, *Didemnum duplicatum* and *Aplidium* cf. *antillense*, were recorded in three of the four northern marinas, respectively. Another six species were recorded solely in southern marinas but only one, *Ecteinascidia turbinata*, was recorded in more than

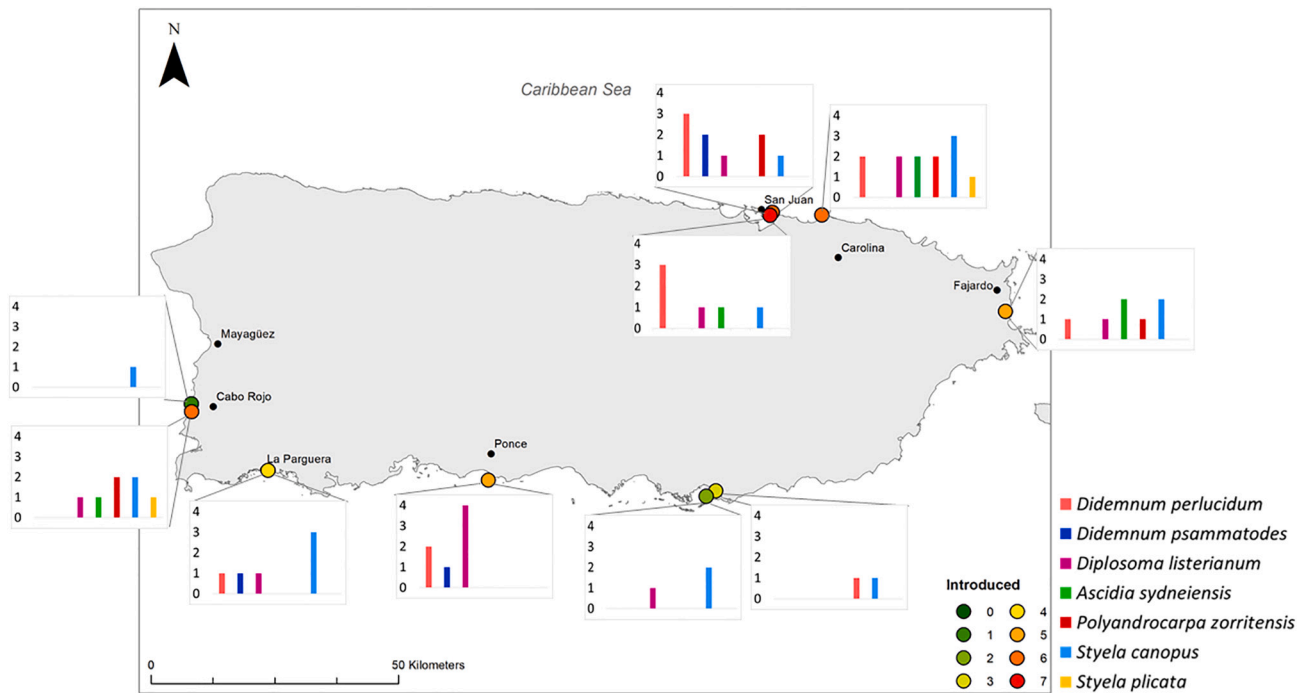


Fig. 3. Map of Puerto Rico depicting the number of introduced species in each marina (colored circles) and relative abundance of the seven introduced species with wide-spread distributions (inset bar graphs, 0 = absent, 1 = rare, 2 = common, 3 = abundant, 4 = very abundant).

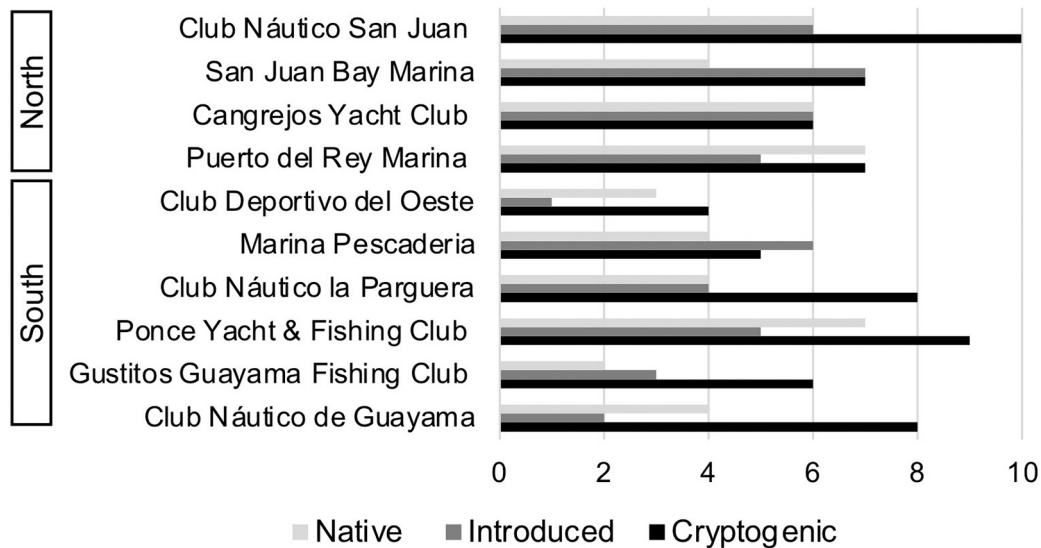


Fig. 4. The number of native (light gray bars), introduced (gray bars), and cryptogenic (black bars) species identified at each surveyed marina. Marinas are grouped by geographic location: North (top) and South (bottom).

two of the six southern marinas.

The similarity of ascidian communities among marinas did not differ significantly between regions (North vs. South) based on presence-absence (PERMANOVA,  $P = 0.234$ ) and relative abundance data ( $P = 0.127$ ; Fig. 6). Similarly, Mantel tests comparing geographic distances between marinas and ascidian community dissimilarity did not show a significant correlation for presence-absence or relative abundance datasets ( $P = 0.345$  and  $P = 0.158$ , respectively). The similarity of ascidian communities among marinas did differ significantly among small, medium, and large marinas (Fig. 6) based on presence-absence data ( $P = 0.043$ ), and relative abundance data ( $P = 0.030$ ). Pairwise analysis did not show significant differences among marina size classes for presence-absence data (all  $P > 0.05$ ) but medium and small marinas

were significantly different ( $P = 0.049$ ) for relative abundance data.

Similar structuring factors were revealed when investigating the communities of introduced species in marinas. Region (North vs. South) as a factor did not show a clear clustering pattern in nMDS plots based on presence-absence or relative abundance data (Fig. 7) and results from PERMANOVA analysis were not significant ( $P = 0.441$  and  $P = 0.274$ , respectively). Communities of introduced species did cluster by marina size in nMDS plots (Fig. 7) and PERMANOVA analysis revealed a significant effect of marina size on introduced ascidian community similarity for both relative abundance data ( $P = 0.003$ ) and presence-absence data ( $P = 0.047$ ).

Four marinas were potential hotspots for invasion and had greater than 25% introduced ascidian species (Fig. S1). San Juan Bay and



**Table 2**

Ascidian species recorded in the surveyed harbors and marinas. Introduction status of each species (native, introduced, or cryptogenic) and number of marinas where the species was observed. GenBank accession numbers for COI sequences are also indicated. NA: Non-applicable; for origin: samples not identified to the species level, and for GenBank acc. Numbers: samples without a COI sequence.

Order	Species	Origin	# Marinas	GenBank acc. numbers	
Aplousobranchia	<i>Didemnum cf. cineraceum</i>	Introduced	1	MT637980	
	<i>Didemnum conchylatum</i>	Native	1	MT637984	
	<i>Didemnum duplicatum</i>	Native	3	MT637936	
	<i>Didemnum perlucidum</i>	Introduced	6	MT637962	
	<i>Didemnum psammotodes</i>	Introduced	3	MT637951	
	<i>Didemnum sp. 1</i>	NA	1	NA	
	<i>Diplosoma listerianum</i>	Introduced	8	MT637946	
	<i>Diplosoma sp. 1</i>	NA	3	MT637954	
	<i>Diplosoma sp. 2</i>	NA	1	MT637965	
	<i>Lissoclinum fragile</i>	Cryptogenic	1	MT637953	
	Didemnidae 1	NA	2	NA	
	<i>Trididemnum savignii</i>	Cryptogenic	2	MT637973	
	<i>Aplidium cf. antillense</i>	Native	3	MT637955	
	<i>Aplidium sp. 1</i>	NA	1	NA	
	<i>Polyclinum constellatum</i>	Cryptogenic	9	MT637964	
	<i>Eudistoma cf. capsulatum</i>	Native	1	NA	
	<i>Eudistoma olivaceum</i>	Native	1	MT637952	
	<i>Eudistoma sp. 1</i>	NA	1	NA	
	<i>Distaplia bermudensis</i>	Cryptogenic	8	MT637947	
	<i>Distaplia styliifera</i>	Cryptogenic	3	NA	
	<i>Clavelina oblonga</i>	Native	4	MT637945, -63, -81	
	Aplousobranchia 1	NA	1	NA	
	Phlebobranchia	<i>Ecteinascidia conklini</i>	Native	6	MT637956, -82-3
		<i>Ecteinascidia turbinata</i>	Cryptogenic	3	MT637966-7
		<i>Ecteinascidia sp. 1</i>	NA	1	MT637957
		<i>Perophora viridis</i>	Native	6	MT637971-2
		<i>Ascidia interrupta</i>	Native	5	NA
<i>Ascidia sydneyensis</i>		Introduced	4	MT637975	
<i>Phallusia nigra</i>		Cryptogenic	6	MT637938, -58	
<i>Phallusia cf. philippinensis</i>		Introduced	2	MT637937	
<i>Rhodossoma turcicum</i>		Introduced	4	MT637968-70	
Stolidobranchia		<i>Botrylloides niger</i>	Cryptogenic	10	MT637960-1
		<i>Botryllus primigenus</i>	Introduced	1	MT637977
		<i>Polyandrocarpa zorritensis</i>	Introduced	5	NA
		<i>Polycarpa spongiabilis</i>	Native	6	MT637949
	<i>Styela canopus</i>	Introduced	9	MT637944, -59, -74, -88	
	<i>Styela sp. 1</i>	NA	1	NA	
	<i>Styela plicata</i>	Introduced	2	NA	
	<i>Symplegma brakenhielmi</i>	Cryptogenic	9	NA	
	<i>Symplegma rubra</i>	Native	9	MT637942-3	
	<i>Symplegma viride</i>	Native	2	MT637950, -79	
	<i>Symplegma sp. 1</i>	NA	1	MT637978	
<i>Herdmania pallida</i>	Cryptogenic	5	MT637941, -89		
<i>Microcosmus exasperatus</i>	Cryptogenic	9	MT637939, -48, -85-7		
<i>Microcosmus helleri</i>	Cryptogenic	2	NA		
<i>Microcosmus sp. 1</i>	NA	1	MT637940		
<i>Pyura vittata</i>	Cryptogenic	3	MT637976		

Cangrejos Yacht Club both on the northern coast of the island exhibited greater than 30% introduced species (35% and 31.6%, respectively). In the South, Marina Pescaderia was the harbor with the highest percentage of introduced species (37.5%), followed by Gustitos Guayama Fishing Club with 27.3% of the identified species classified as introduced.

#### 4. Discussion

This study documented the presence of 47 ascidian species in 10 harbors and marinas around the coast of Puerto Rico during March 2019. Of the 25 species described in mangroves by Toffart (1983), 14 were retrieved by both Van Name (1930, 1945) and our study. *Ecteinascidia turbinata* was not recorded by Van Name (1930, 1945) but was cited by Toffart (1983) and observed here. Of the 27 species recorded in Puerto Rico by Van Name (1930, 1945), 20 were observed during the present study, including five of the species considered introduced to the Caribbean: *Diplosoma listerianum*, *Ascidia sydneyensis*, *Rhodossoma*

*turcicum*, *Styela canopus*, and *S. plicata*, and nine now considered cryptogenic: *Trididemnum savignii*, *Polyclinum constellatum*, *Distaplia bermudensis*, *Phallusia nigra*, *Botrylloides niger*, *Symplegma brakenhielmi*, *Microcosmus exasperatus*, *M. helleri*, and *Pyura vittata*. *Botrylloides niger*, classified as native in this study, was observed by Van Name (1930) and was present in all 10 harbors investigated here. Seven introduced species with worldwide distributions were also found in Puerto Rico: *Didemnum perlucidum*, *D. psammotodes*, *Diplosoma listerianum*, *Ascidia sydneyensis*, *Polyandrocarpa zorritensis*, *Styela canopus*, and *S. plicata*. *D. listerianum*, *A. sydneyensis*, and *S. canopus* were previously recorded in Puerto Rico (Van Name, 1930; Toffart, 1983). *Styela plicata*, recorded by Van Name (1930) as abundant, was rare in this study and only found twice: a tiny specimen in Cangrejos Yacht Club and another equally small individual in Marina Pescaderia.

In total, we found 20 species more than in Van Name (1930, 1945), including the first recorded occurrence in the Atlantic of *Phallusia cf. philippinensis*. Except for a slightly clearer tunic (black-brown to gray), the species is morphologically similar to *P. nigra* and may have been

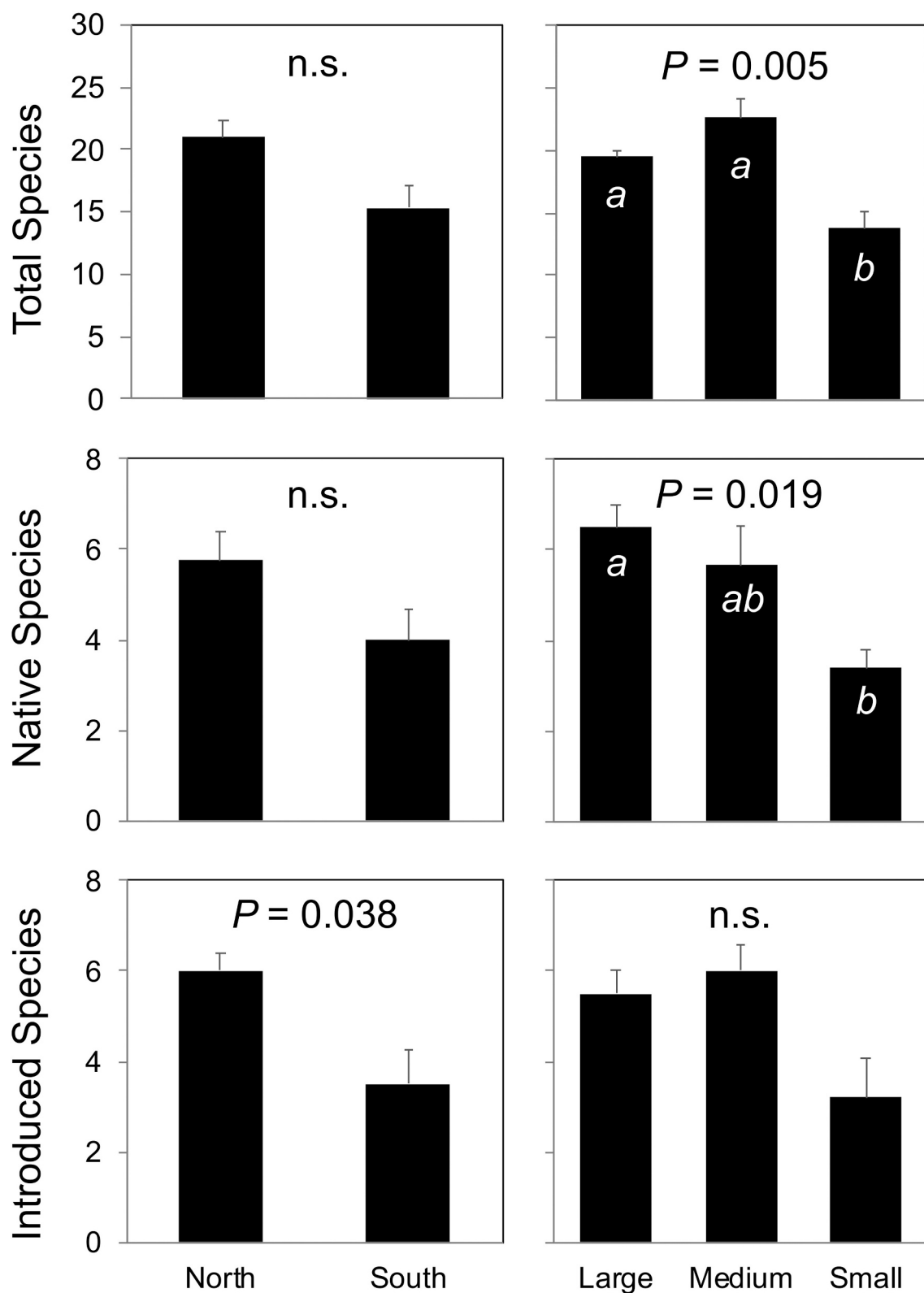


Fig. 5. Richness of ascidian communities across geographic locations (North vs. South) and harbor sizes (Large >200 boat slips, Medium = 100–199 boat slips, Small <100 boat slips). Data for all 47 ascidian species (entire dataset) and subsets of only native species and introduced species are shown. P-values are shown for significant *t*-test and ANOVA outcomes (n.s. = not significant). Different letters on bars denote significantly different pairwise means. Error bars represent ±1 standard error (SE).



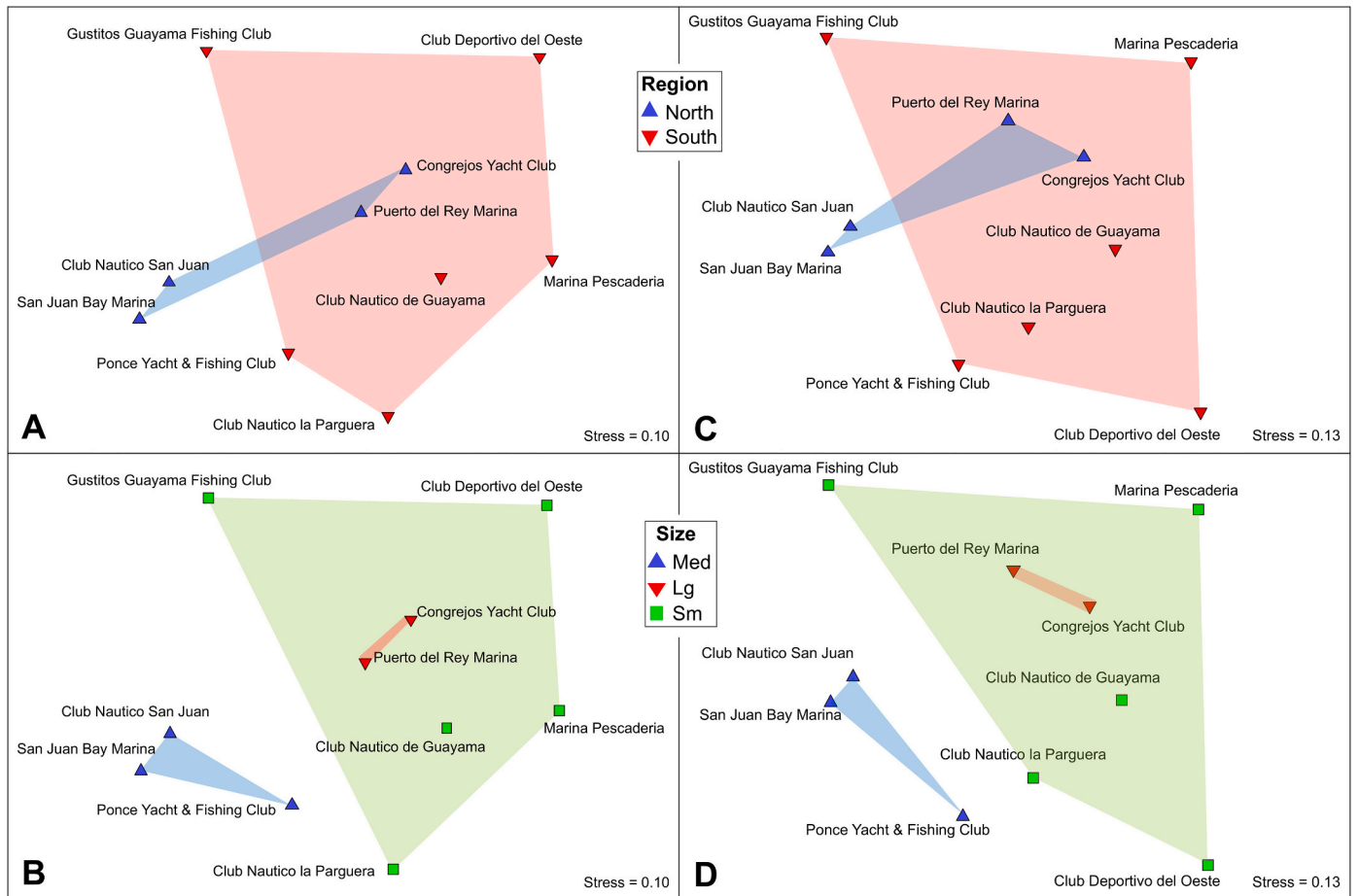


Fig. 6. Non-metric multidimensional scaling (nMDS) plots comparing communities of ascidian species in marinas grouped by region (A, C) and marina size (B, D). Visualizations based on Bray-Curtis similarity values calculated from presence-absence data (A, B) and relative abundance data (C, D).

misidentified in the Caribbean as it was for Japan and Hawaii (Vandepas et al., 2015). Eleven of the 47 species were identified only to the genus level or above but were clearly distinct from the others based on morphology or genetic characterization. The remaining 36 species were classified as: 11 introduced, 13 cryptogenic, and 12 native in the area. Geographic location and distance between harbors did not have significant impacts on the composition of ascidian communities, while marina size affected both richness and community composition. Interestingly, marina size had different effects on native and introduced species communities, with native species exhibiting greater richness in larger marinas and introduced species exhibiting compositional differences across marina sizes. Thus, marina size was more important in determining ascidian community structure in Puerto Rico than distance among marinas.

The 11 introduced species in this survey included six not previously described in Puerto Rican waters by Van Name (1930, 1945): *Didemnum perlucidum*, *D. psammotodes*, *D. cf. cineraceum*, *Phallusia cf. philippinensis*, *Botryllus primigenus*, and *Polyandrocarpa zorritensis*. Four cryptogenic species found during the present survey but not recorded by Van Name (1930, 1945) may represent additional introductions: *Lissoclinum fragile*, *Distaplia stylifera*, *Ecteinascidia turbinata*, and *Herdmania pallida*. With ship traffic increasing globally, new introductions are expected to occur. The number of introductions to Puerto Rico in the 90 years since the Van Name (1930) study is well within the expected rate described by Cohen and Carlton (1998).

Seven of the introduced species also have global distributions: the aplousobranchs *Didemnum perlucidum*, *D. psammotodes*, and *Diplosoma listerianum*, the phlebobranch *Ascidia sydneyensis*, and the stolidobranchs

*Polyandrocarpa zorritensis*, *Styela canopus*, and *S. plicata*. The origin of *Didemnum perlucidum* is currently unknown; it was first described as a new species by F. Monniot (1983a) in Guadeloupe, where it was found exclusively on artificial substrates. There is no species matching *D. perlucidum* in Van Name (1930, 1945), although the author did not sample artificial substrates other than wharf pilings in Guanica Bay. The COI sequence obtained here matched to a GenBank sequence for this species from western Australia (JQ731735, 99.82% identity; Smale and Childs, 2012). The mud-like *Didemnum psammotodes* is native to Australia and the Indo-Pacific (Monniot and Monniot, 1985, 1994; Carlton and Eldredge, 2009) but is now commonly observed in the Caribbean (Bingham, 1992; Rocha and Monniot, 1995; Rocha et al., 2005). The best match for the Puerto Rican *D. psammotodes* COI sequence corresponded to *D. psammotodes* from Brazil (KU221189; Oliveira et al., 2017); however, only 89.49% sequence identity was recovered (98% coverage). Low identity match between the Oliveira et al. (2017) sequence and ours could result from either high genetic variability for this locus and species (as observed in other aplousobranchs, e.g. *Cystodytes dellechiaiei*; López-legendil and Turon, 2006) or a completely different geographic origin for the specimens found in Puerto Rico.

*Diplosoma listerianum* is often observed overgrowing other organisms. This species was described from the northeastern Atlantic (Milne-Edwards, 1841; Berrill, 1950) and is now one of the most widely distributed marine invertebrates (Willis et al., 2011; Pérez-Portela et al., 2013). The COI sequence obtained from the Puerto Rican specimen corresponded to the largest clade of all, 'clade A', with the highest genetic diversity and widest distribution, including the Mediterranean Sea,

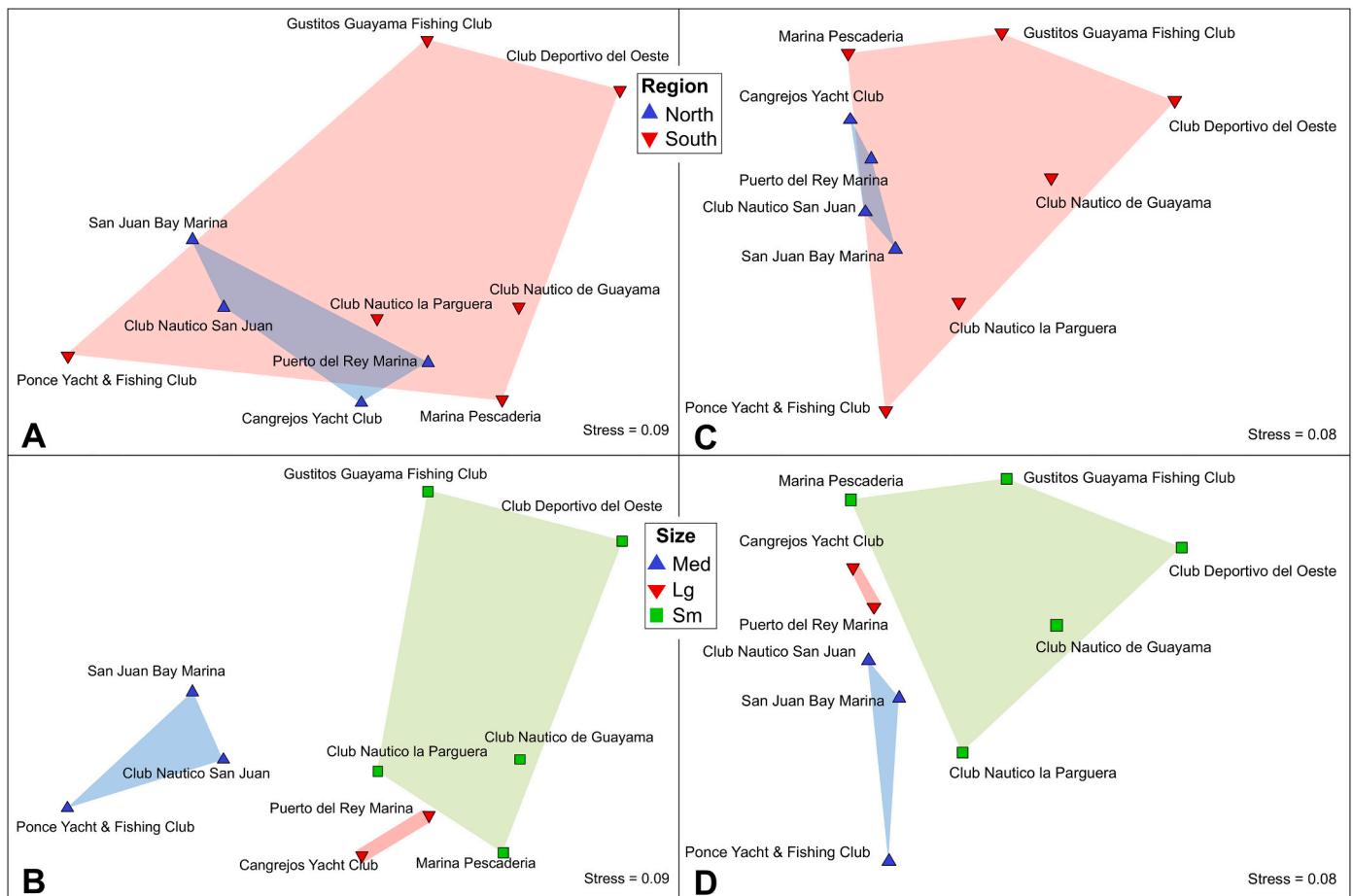


Fig. 7. Non-metric multidimensional scaling (nMDS) plots comparing communities of introduced species in marinas grouped by region (A, C) and marina size (B, D). Visualizations based on Bray-Curtis similarity values calculated from presence-absence data (A, B), and relative abundance data (C, D).

and the Atlantic, Pacific and Indian Oceans (Pérez-Portela et al., 2013). Specifically, our COI sequence was 100% identical (93% coverage) to *D. listerianum* haplotype 18 (KF791884) within subclade A1 (Pérez-Portela et al., 2013). Subclade A1 was formed by sequences obtained from specimens strictly from the Caribbean, with the exception of a couple of haplotype 18 sequences from specimens collected in South Africa (Pérez-Portela et al., 2013). Thus, sequence data suggests that at least some individuals of South African *D. listerianum* share a genetic signature that includes the Puerto Rican haplotype.

The phlebobranch *Ascidia sydneiensis* was first described from Sydney, Australia, by Stimpson (1855). This species was already recorded by Van Name (1930), indicating that it has been a resident in the island for at least 90 years. The single COI sequence obtained here matched a sequence for *Ascidia virginea* (98.57% identity, 100% cover) collected from a harbor in southern Catalonia, Spain (KF309647; López-Legentil et al., 2015). *A. virginea* is considered native to Europe and to date there is no indication of this species spreading anywhere else. *A. virginea* and *A. sydneiensis* are easily distinguishable morphologically based on differences in the musculature, and the widely separated siphons and greatly expanded posterior intestine in *A. sydneiensis*. Surprisingly, no phylogenetic study for the genus *Ascidia* exists. Thus, at this point we can only hypothesize that the COI gene for these two species (and possibly for the genus) evolves at a slower pace, resulting in sequence identities between these species <3%.

The stolidobranch *Polyandrocarpa zorritensis* was first described by Van Name (1931) from a harbor in Peru and now exhibits a worldwide distribution, including Panama, the southeastern and southwestern USA, Hawaii, Galápagos, Italy, and Spain (Turón and Perera, 1988;

Lambert and Lambert, 2003; Brunetti and Mastrototaro, 2004; Carman et al., 2011; Villalobos et al., 2017; Lambert, 2019). Unfortunately, we were unable to obtain a COI sequence for this species and thus we are unable to suggest potential origins of the Puerto Rican specimens. *Styela canopus* is now widely distributed in temperate and tropical coastal waters worldwide, especially in harbors (Kott, 1998; Lambert and Lambert, 2003; Lambert, 2003; Salgado-Barragan et al., 2004; Trott, 2004; Lambert et al., 2005). Here, we obtained four COI sequences for *Styela canopus*: one from Cangrejos Yacht Club, two from Club Náutico de Guayama, and one from Puerto del Rey Marina that resulted in 3 haplotypes. The best GenBank matches for all 3 haplotypes were sequences obtained for *Styela gibbsii* from northwestern USA and Canada (identity >80%, cover >98% in all cases) and *S. canopus* from Brazil and Virginia, USA (identity >79%, cover >96%). Although percent identities are low, *S. canopus* appears to be a species with an unusually high genetic variability for the COI gene (over 141 haplotypes reported in GenBank so far), highlighting a need for both phylogenetic and phylogeography studies for the species. Finally, *Styela plicata* was reported as a very abundant species in Puerto Rico by Van Name (1930); however, only two tiny individuals were observed during our survey, one in Cangrejos Yacht Club and the other in Marina Pescaderia. *S. plicata* is a species that, when it is present, usually occurs in great numbers (Barros et al., 2009; Pineda et al., 2011, 2016; Villalobos et al., 2017), can tolerate pollution (Galletly et al., 2007; Pineda et al., 2012a), temperature and salinity changes (Thiyagarajan and Qian, 2003; Pineda et al., 2012b), and is able to reproduce year-round (Pineda et al., 2013). Given those attributes, we have no ready explanation of what may have caused the sharp decrease of this species in Puerto Rico.

Geographic location (North vs. South of the island) did not have a large effect on ascidian community composition, a result that was somewhat unexpected given climate differences among both regions and international shipping routes. The northern half of Puerto Rico is mostly a rainforest type environment, experiencing significantly more rainfall than the southern half, which is semi-arid (Gómez-Gómez et al., 2014). Runoff from rainfall can decrease the salinity in coastal environments, rendering these habitats inhospitable for the long-term establishment of most if not all ascidian species (Pineda et al., 2012a, 2012b; Rocha et al., 2017), as was the case for Club Náutico de Arecibo. On the other hand, northern harbors with established ascidian communities were the largest in the area. Marinas around San Juan are in close proximity to ports receiving large international vessels (e.g., cruise ships and cargo vessels), and Puerto del Rey marina is the largest marina in the entire Caribbean (1000 slips). Accordingly, we expected northern harbors to host more diverse communities of introduced species. The total number of introduced species was indeed significantly higher for the northern harbors than the southern ones, with similar though non-significant trends in terms of species richness overall and for native species. At the community level, these differences in individual ascidian species were not sufficient to impact compositional similarity between regions. Pairwise geographic distances between harbors and ascidian community similarity were not significantly correlated: harbors in closer proximity often hosted very different ascidian communities. Thus, overall differences in climatic conditions and international ship traffic between the North and the South of the island manifested in some differences in ascidian richness but no significant differences in overall community composition.

Marina size did have a significant effect on overall species richness and presence of native ascidians. Large and medium harbors consistently exhibited greater richness than small harbors, with significant pairwise differences between medium and small marinas when considering all species, and between large and small marinas when considering only native species. The number of introduced species present at each marina was independent from its size, although in general smaller marinas tended to have fewer introduced species. To date, few studies have included harbor size as a potential factor influencing ascidian species richness and distribution among harbors. López-Legentil et al. (2015) measured dock length as a proxy of harbor size and performed a linear regression analysis with the total number of species recorded. As in the present study, the authors found a significant relationship between harbor size and species richness, but harbor size only explained 17.2% of the observed variance (López-Legentil et al., 2015). Thus, other biotic and abiotic factors such as patterns of secondary spread dictated by local traffic (Wasson et al., 2001; López-Legentil et al., 2015), salinity and pollution, may also contribute to determine species compositions and abundances in Puerto Rico.

As far as we know, there are no studies characterizing ascidian communities in harbors and marinas of other Caribbean Islands. Such studies would have helped us determine the relative impact and prevalence of species introductions in Puerto Rico. In the Pacific, two of the largest island harbors were monitored for ascidian introductions: Apra Harbor in Guam (22 cryptogenic and 9 introduced species; Lambert, 2002), and Pearl Harbor in Hawaii (9 introduced and 1 cryptogenic species; Coles et al., 1999). These numbers are within the range of what we found here: 13 cryptogenic and 11 introduced species in total (10 cryptogenic and 8 introduced in just the San Juan area), and call for equivalent management policies. In addition, all harbors visited here were shown to have more introduced and cryptogenic species than natives. The prevalence of introduced over native species is a common occurrence in harbors and marinas worldwide (Lambert and Lambert, 1998, 2003; Marins et al., 2010; Tracy and Reynolds, 2014; López-Legentil et al., 2015) and is attributed to these species' higher resilience to pollution (Piola and Johnston, 2008), warmer and fluctuating seawater temperatures (Yamaguchi, 1975; Rocha, 1991; Stachowicz et al., 2002), and fluctuations in salinity (Lambert and Lambert, 1998). Since

introduced species often outcompete natives, it is of utmost importance to learn more about their life cycles and environmental tolerance thresholds to elucidate potential patterns of spread and the likelihood of any of these species to become invasive (see Lambert, 2002, 2005). Likewise, periodic surveys of species diversity and abundances in harbors and marinas, as well as the surrounding natural habitats, should be conducted to record the arrival of new species and monitor the spread of established ones in an effort to protect coastal environments from detrimental species.

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## CRediT authorship contribution statement

**Olivia T. Streit:** Investigation, Formal analysis, Visualization, Writing – original draft. **Gretchen Lambert:** Methodology, Investigation, Formal analysis, Validation, Visualization, Writing – review & editing. **Patrick M. Erwin:** Conceptualization, Methodology, Investigation, Formal analysis, Validation, Visualization, Writing – review & editing, Supervision, Funding acquisition. **Susanna López-Legentil:** Conceptualization, Methodology, Investigation, Formal analysis, Resources, Validation, Visualization, Writing – review & editing, Supervision, Funding acquisition, Project administration.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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