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## Rapid establishment of the non-indigenous ascidian *Styela plicata* and its associated bacteria in marinas and fishing harbors along the Mediterranean coast of Israel

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### Abstract

*Styela plicata* (Lesueur, 1823) (Tunicata; Stolidobranchia; Styelidae) is a solitary ascidian with a global distribution. Until recently it had not been observed along the Mediterranean coast of Israel, but is now to be found in many harbors attached to suspended ropes and other artificial structures. In order to monitor the populations of *S. plicata*, we surveyed eight harbors along the Israeli Mediterranean coast. Our findings demonstrate that the condition and level of maintenance of each harbor significantly affect the presence of *S. plicata*. We also characterized the microbial communities in the tunic of three individuals using 16S rRNA gene tag pyrosequencing and compared them to those in the surrounding seawater, in order to determine whether their symbiotic bacteria contribute to the successful establishment of this ascidian species. We found 15 bacterial phyla in total, of which 14 were present in the animal hosts; six were present in all of the individuals and four in two of them. Three of the 15 phyla observed were absent in the surrounding seawater. The high variability in the microbial communities among the three hosts suggests a mechanism of horizontal transmission and may play a contributing role in the successful invasion of new and less tolerant niches.

**Keywords:** Ascidians, marine bioinvasions, microbial diversity, Mediterranean Sea.

### Introduction

Fouling communities are often dominated by ascidians (Lambert & Lambert, 2003). Due to the increasing marine traffic and the growing number of man-made structures at sea, such as rigs, aquaculture constructions, marinas and so forth, ascidians and other non-native species are able to disperse to great distances and inhabit new niches. In addition they have the potential to become invasive and to alter local communities (Lambert, 2007). The Mediterranean Sea is especially susceptible to marine bioinvasions, as it connects to the Atlantic Ocean through the narrow Strait of Gibraltar and to the Red Sea through the Suez Canal, giving rise to the well-documented phenomenon of “Lessepsian migration” of species (Por, 1978). The coastline along the Mediterranean Sea is heavily populated and features many marinas and ports, hosting all boat sizes from small recreational ones to large commercial ships and tankers. A growing awareness of these marine introductions has led to the establishment of several monitoring and management programs in the Mediterranean Sea (Katsanevakis *et al.*, 2011).

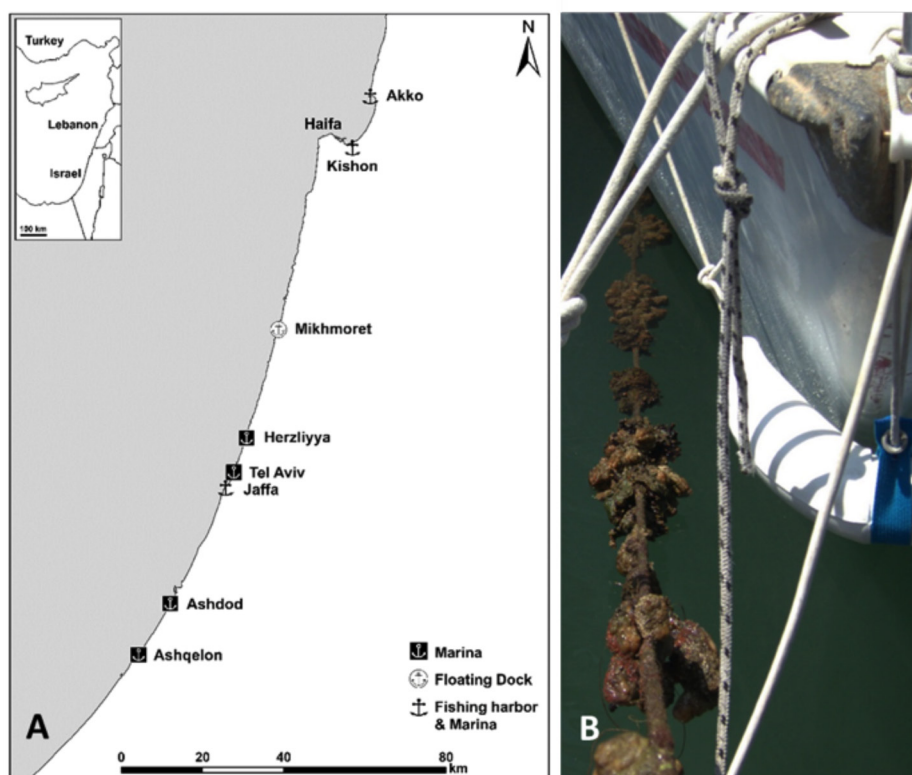
*Styela plicata* (Lesueur, 1823) (Tunicata; Stolidobranchia; Styelidae) is a solitary ascidian with a global distribution. Its origins are no longer traceable, since it probably invaded different regions of the world a very long time ago by attaching to ship hulls (Pineda *et al.*, 2011). *S. plicata* is known to inhabit harbors and marinas around the world, mostly fouling on artificial substrates (Barros *et al.*, 2009; Pineda *et al.*, 2011), with the exception of one reported case in Japan where it was found on a natural substrate (Pineda *et al.*, 2011). *S. plicata* is highly adaptable to changing environments and has a high tolerance for temperature and salinity fluctuations (Pineda *et al.*, 2012).

In Israel, until recently, *S. plicata* had only been observed once, on a natural sandy patch in Haifa Bay (Pérès, 1958). However, its correct identification is doubted as it refers to a tunic filled with sand and does not fit the description of the species given by Lesueur (1823). In the past three to four years there have been an increasing number of reports from fishermen and sailors describing the presence of *S. plicata* on artificial substrates in marinas along the coast of Israel (Shenkar N. pers. obs.). Clusters of *S. plicata* can now be found attached to ropes

suspended in the water or stretched between boats (Fig. 1B). We suspect that the current spread of *S. plicata* in Israel may be following the same pattern described for *Herdmania momus*, which was found first on man-made structures, but now also thrives on natural rocky substrates in northern Israel (Shenkar *et al.*, 2009; Gewing *et al.*, 2014).

As found for other marine invertebrates, ascidians are holobionts, hosting diverse microbial communities in various degrees of symbiotic interaction (Kwan *et al.*, 2012; Erwin *et al.*, 2014; Tianero *et al.*, 2015). Microbial symbionts are thought to be the main source of bioactive natural products occurring in ascidians (Schmidt & Donia, 2010; Schofield *et al.*, 2015), contributing both to the

defensive mechanism of the sessile ascidian and to the metabolic needs of its host (Martínez-García *et al.*, 2008; Erwin *et al.*, 2014; Tianero *et al.*, 2015). Assessing the ascidian holobiont's microbial communities, which may also be involved in the process of occupying new niches, is expected to shed new light on the processes that enable the colonization of new habitats by this species. In this work, we investigated the current distribution of *S. plicata* in relation to the condition and level of maintenance of each harbor, together with a description of the diverse microbial communities inhabiting the tunic of this species in Israel. Finally, we compared them to the bacterial communities described for this species in Spain (Erwin *et al.*, 2013).



**Fig. 1:** A. Israel Mediterranean coastline showing location of marinas and fishing harbors visited in this survey. Harbor types were divided into groups based on the major activity in each one. Map was produced using ArcGIS 10.0 (distributed by Esri). B. *Styela plicata* clusters attached to ropes stretched between small boats in Jaffa Port.

## Materials and Methods

### *Styela plicata* survey

Eight marinas and fishing harbors were visited between the months of March and May 2015, when sea surface temperature ranged between 18 and 23 °C. The sites were divided into two groups, based on their main purpose (Fig. 1A): small fishing harbors hosting active fishing boats (group A); and marinas - utilized mainly for recreational purposes and hosting mostly yachts (group B). Out of the four designated marinas, three were built

in the mid-1990s, while the Tel Aviv marina was built two decades earlier but was renovated during the last decade and is used for recreational purposes; Akko and Jaffa, in contrast, are old cities each with an old existing port (now used mainly as a fishing harbor); the Kishon “Shavit” harbor was built about 50 years ago to serve as the main international fishing port of Israel; and, finally, Mikhmoret, which is a small floating dock marina and is unique in this sense.

We checked for the presence of *S. plicata* on suspended ropes and chains connected to the docks and boats

(Fig. 1B). Several sampling stations were planned for each harbor (from hereon, when referring to both marinas and fishing harbors, only the word harbor will be used), to represent different current regimes within the harbor, or proximity to the sea. Originally, 10 ropes were to be inspected at each station. However, as the number of ropes varied among harbors and was insufficient in some of the stations, we decided to pool the results. Hence, each harbor represents one site. Boat hulls and other submerged structures were also checked visually but not quantitatively due to technical limitations. Statistical analysis was carried out using GraphPad Prism version 6.01 for Windows (GraphPad software, www.graphpad.com) and R, version 3.2.4 (R core team 2016, www.R-project.org). Data were normalized using Prism. One-way analysis of variance (ANOVA) was used to determine whether any statistical differences existed among the means of the number of specimens on ropes in the different harbors. Tukey post-hoc analysis was used to determine which means differ significantly from others.

### **Sample collection and processing for microbial diversity assessment**

Three individuals of the solitary ascidian *S. plicata* were collected from the fishing port of Jaffa (32.05175° N, 34.749472° E) on July 30<sup>th</sup>, 2014, by detaching individuals from suspended ropes and placing them in sterile plastic bags. Three 500 ml seawater samples were also collected from the same location in separate bags, providing background data on the surrounding microbial community (water salinity was 35 ppt at the time of sampling). Animals were transferred to the lab in a cooler within one hour of capture. In the lab, animals were washed three times with 0.2µM filtered seawater to remove non-intrinsic microbes. A piece of the inner tunic was removed using a sterile scalpel and scissors and stored at -80 °C for later DNA extraction. Water samples were filtered through 0.2µM polycarbonate filters (PCTE filters, Maine Manufacturing, ME, USA). Filters were stored at -80 °C.

### **DNA extraction and data processing**

DNA extractions were performed using the Power Plant DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), for both tissue and water samples (filters). To ensure the 16S rRNA gene amplification, DNA was amplified using the universal primers 63F (5'- CAG GCC TAA CAC ATG CAA GTC -3') and 1387R (5'- GGG CGG WGT GTA CAA GGC -3') (Marchesi *et al.*, 1998). Both DNA and PCR products were visualized on 1% agarose gel to ensure quality and presence of 16S rRNA amplicons.

DNA samples were then sent to MR DNA (Shallowater, TX, USA) for Illumina MiSeq sequencing. The V4 region of the 16S rRNA gene was amplified using the primer set 515F (5'- GTG CCA GCM GCC GCG GTA

A -3') and 806R (5'- GGA CTA CHV GGG TWT CTA AT -3') (Caporaso *et al.*, 2012). Amplicons were purified, pooled in equal proportions, and a library was prepared following Illumina TruSeq DNA library preparation protocol. Processing of sequence data was done following the pipeline described in Weigel & Erwin (2015) in mothur (Schloss *et al.*, 2009). In brief, raw sequence reads were filtered for ambiguous base calls (maxambig = 0), amplicon size (maxlength = 300, minlength = 200), barcode mismatches (bdiffs = 0), primer mismatches (pdiffs = 2), and homopolymers (maxhomop = 8). The resulting sequences were aligned with the Greengenes reference database (gg\_13\_5\_99) and trimmed to the V4 region. Further analysis removed putative chimeras based on the UChime algorithm implemented in mothur. Sequences were taxonomically assigned and grouped into operational taxonomic units (OTUs) at 97% sequence similarity. Sampling depth ranged from 12,656 to 132,859 sequence reads per sample; thus each dataset was subsampled to the lowest read count (n = 12,656).

To compare the overall bacterial community structure between the animal and the surrounding water column, we performed a PERMANOVA analysis of Bray-Curtis dissimilarities, and applied a Monte Carlo simulation to correct for small sample size. Analysis was performed using E-Primer (version 6.1.11; Primer-E Ltd., Plymouth, UK).

## **Results and Discussion**

The current study documents the rapid spread of *Styela plicata* in harbors along the Mediterranean coast of Israel. Since its first unconfirmed report in 1958 by Pérès, it had not been documented again until now. Following meticulous surveys of eight harbors, we are now able to suggest a potential common attribute of harbors hosting aggregates of *S. plicata* on ropes, or hosting large numbers of this organism. Our finding could potentially contribute to the future effective management and control of this species.

### ***Styela plicata* survey**

Survey results are presented in Table 1. There was a significant difference in the means of *S. plicata* numbers among harbors as determined by one-way ANOVA (F (6, 208) = 7.499, P < 0.0001). A Tukey post-hoc test revealed that in Jaffa harbor, the number of individuals was significantly higher than in Kishon harbor (-5.274 min diff, P < 0.0001), Herzliyya harbor (-5.594 min diff, P < 0.0001), Tel Aviv harbor (-5.522 min diff, P < 0.0001), Ashdod harbor (4.194 min diff, P = 0.0007), and Ashqelon harbor (3.385 min diff, P = 0.0488). There was no statistically significant difference among the other harbors and no significant difference between Jaffa and Akko. In terms of boats and fishing activity, the main commercial fish-

ing activity is carried out in Jaffa (47 boats, 12 commercially active), Akko (35 boats, 15 commercially active), and Ashqelon (35 boats, 8 commercially active). Kishon “Shavit” harbor features the highest number of boats (over 70), but many of these are not active. Ashdod also has a designated fishing area that is not active commercially (Malamud *S. pers. comm.*).

When comparing the average number of individuals per rope, which does not take into account the number of individuals on each separate rope, Jaffa harbor was found to host a higher number of *S. plicata* in comparison with other sites, with an average of  $5.6 \pm 1.5$  individuals per rope ( $n = 32$ ). It is important to note that at this particular site, it was very common to find *S. plicata* as aggregates of up to roughly 25 individuals, unlike at the other locations with the exception of Akko (Fig. 1B), thus leading to a high abundance per individual rope. Akko, Ashqelon, and Ashdod exhibited a lower abundance of *S. plicata* per rope, with an average of  $3.1 \pm 2.7$  for Akko ( $n = 15$ ),  $2.2 \pm 0.8$  for Ashqelon ( $n = 24$ ), and  $1.4 \pm 0.5$  for Ashdod ( $n = 40$ ). However, the sampling effort, as reflected in the number of ropes pooled, presents a different pattern for each harbor: whereas Akko has very few ropes in the water and aggregates of *S. plicata* on some of the ropes (similar to Jaffa), more ropes were examined in Ashqelon and Ashdod (15 ropes for Akko, versus 24 and 40, respectively, Table 1). Both of these sites are relatively new marinas and were initially classified as group B (yacht marinas). Ashqelon is closer in its activity profile to Akko than to Ashdod, and has a very active commercial fishing wharf, which also exhibited the highest abundance and diversity of other invertebrates. Ashdod has many ropes in the water, facilitating the settlement of larvae, but is less active in terms of fishing activity. Akko presented both a high degree of biodiversity and high numbers of ascidians, including aggregates of *S. plicata*. Some of these ascidians and other invertebrates in Akko were impossible to reach or add to the survey, as they were located on poles and on cage traps deployed by fishermen, one to two meters below the water surface. In this, as well as in the main use of Akko as an old fishing harbor, it resembles Jaffa harbor.

The other four harbors presented much lower numbers of *S. plicata* individuals per rope, with “Shavit” marina leading with higher numbers compared to Tel Aviv, Herzliyya, and Mikhmoret (Table 1). “Shavit” is the international fishing port of Israel and has wharfs for larger fishing vessels as well as many smaller docking areas for yachts. As such, due to the higher rate of international traffic, we had expected to observe high numbers of ascidians and other invasive species in this harbor, arriving from the Levant basin and even farther. We suggest that the reason for the small numbers of *S. plicata* found in “Shavit” lies in the high level of regularly performed maintenance work there, such as cleaning vessels and docks and pulling ropes out of the water. There were relatively few ropes

to pull and these were concentrated in the area of the fishing wharfs, rather than the yacht area of the harbor.

Tel Aviv and Herzliyya are well-maintained in terms of cleaning the docks and water and have no commercial fishing activity. It is noteworthy that in the Herzliyya marina, which contains various docking areas and an inner lagoon with minimal water exchange, no ascidians were found. Mikhmoret is a small private sailboat marina, consisting of floating docks only. Sailing activity is close to shore and rarely international. No marine invertebrates were spotted on the ropes or docksides. This is in contrast to Eilat, where the floating docks exhibited high numbers of ascidians on the sides and bottoms of the docks, including *H. momus*, which has also invaded the natural environment of northern Israel (Koplovitz *et al.*, 2016). In conclusion, our data demonstrate that a low level of maintenance together with high activity in a marina, greatly contribute to the successful establishment of *S. plicata*.

### **Microbial community of *S. plicata***

Previous research on microbial associates was carried out on *S. plicata* (Erwin *et al.*, 2013) and other ascidians (Erwin *et al.*, 2014; López-Legentil *et al.*, 2015, 2016; Tianero *et al.*, 2015). In the present study, 16S rDNA sequences were obtained from three *S. plicata* individuals and from three water samples. The microbial phyla presented in Figure 2 refer to the three individuals separately and to the average of the three water samples.

Our taxonomically-assigned analysis revealed 15 different bacterial phyla, of which 12 phyla were present in the water samples and 14 in the ascidian samples (Fig. 2 & Table S1).

Only five bacterial phyla demonstrated more than 1% relative abundance in the animal, and in only one individual of the three analyzed hosts. When all three host samples were averaged, only two phyla exhibited a relative abundance greater than 1%. The marine phyla SAR406 was found solely in the water samples, but at less than 0.01%. The three phyla found only in the *S. plicata* samples were the two candidate divisions WS3 and OP3, and *Fibrobacteres*, but these were observed in one host individual only and also at <0.01% of all the present phyla. The major phylum that dominated both water samples and all ascidian samples was *Proteobacteria*, which exhibited 59% and 52% of all reads, respectively. Within this phylum, most taxa were affiliated with *Alphaproteobacteria* (46% and 35% of total bacterial reads in the seawater versus ascidian samples) and *Gammaproteobacteria* (10% and 19%, respectively). The next most dominant group in the water samples was *Bacteroidetes* (33% versus 6% in *S. plicata* samples). However, we observed a large variation among bacterial phyla values present in the three host replicates (Fig. S1 & Fig. 2). *Proteobacteria* represented 93% of OTU reads in one host (sample sp1), versus 8% (sp2) and 54% (sp3) in the two other hosts.

**Table 1.** *Styela plicata* recorded from fishing harbors and marinas along the Mediterranean coast of Israel. Type A harbors represent small fishing harbors; Type B harbors represent recreational marinas hosting mainly yachts; X is a floating dock harbor. Number of ropes refers to pulled ropes.

Location	Description	Type	Latitude/Longitude	<i>S. plicata</i> counts	No. of ropes	Avg. / rope	SE
Akko	Fishing port and marina	A	32.919222° N 35.070278° E	47	15	3.1	2.7
Kishon "Shavit"	Fishing port and marina	A	32.805944° N 35.030528° E	8	25	0.3	0.3
Mikhmoret	Floating docks marina	X	32.402528° N 34.866833° E	0	21	0	0
Herzliyya	Marina	B	32.162639° N 34.796028° E	0	51	0	0
Tel Aviv	Marina	B	32.086278° N 34.767528° E	2	28	0	0
Jaffa	Fishing port and marina	A	32.05175° N 34.749472° E	179	32	5.6	1.5
Ashdod	Marina	B	31.795333° N 34.626889° E	56	40	1.4	0.5
Ashqelon	Marina	B	31.681722° N 34.556556° E	53	24	2.2	0.8

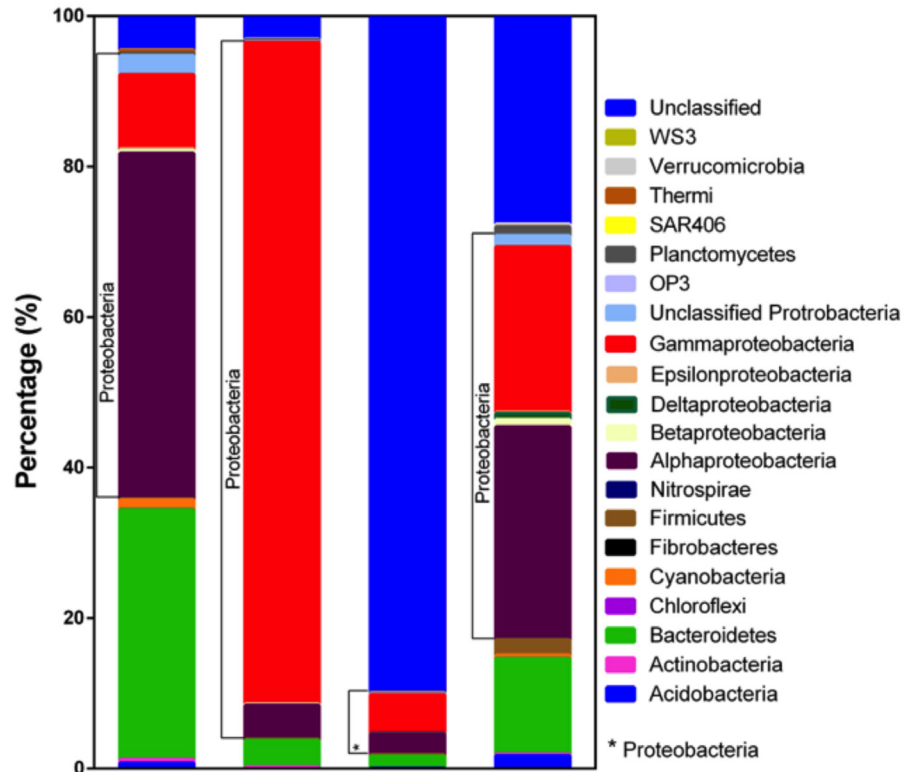
*Bacteroidetes* represented 3.5% of the total OTU reads (sp1), 1.6% (sp2) and 12.7% (sp3). Moreover, about 40% of the reads in the averaged organisms were taxonomically-unassigned, varying from 3% (sp1) to 90% (sp2; 27.6% in sp3) unassigned OTUs. In the water samples, the unassigned groups accounted for less than 5% of all reads. The remaining bacterial phyla were present in <1% of the average reads. These included the following phyla: *Acidobacteria* (<0.1% in sp2, 1.7% in sp3 versus 0.7% in averaged seawater samples); *Actinobacteria* (0.2% in sp1, sp3 versus 0.5% in seawater); *Cyanobacteria* (0.43% in sp3 and less than 0.1% in the other samples, versus 1.3% in seawater); *Firmicutes* (0.1% in sp1, 0.2% in sp2 and 2% in sp3 versus 0.14% in seawater); *Planctomycetes* (0.2% in sp1, 0.1% in sp2 and 1.1% in sp3 versus 0.3% in seawater); and *Verrucomicrobia* (0.1% in sp1, <0.1% in sp2 and 0.4% in sp3 versus less than 0.1% in seawater samples). A comparison of community structure between the animal and the surrounding seawater revealed significant differences ( $P = 0.033$ , Fig. S1).

Following a BLASTn search against specific unassigned OTUs that had appeared in the host samples but were rare to non-existent in the water samples, we noticed the presence of *Chloroflexi* as a dominant phylum in the host. This phylum was present in all three samples (Table S2), in a much higher number of reads in sample sp2 and in similar numbers of reads in samples sp1 and sp3. Other hits for OTUs present in the individual hosts but not in the water samples correlated to the two dominant groups, *Proteobacteria* and *Bacteroidetes* or to environmental uncultured bacteria (Table S2).

*S. plicata* from Spain (Erwin *et al.*, 2013) has been demonstrated to host similar bacterial phyla as found in the Israeli samples, with a core community of *Proteobacteria* (*Alpha* and *Gamma*) and *Bacteroidetes* as the dominant groups (Table S1). These bacterial phyla are also known to be prevalent in other ascidians (Erwin *et al.*, 2014). The third dominant group in the samples from Spain was *Planctomycetes*, which was present in the three Israeli hosts at a higher prevalence than in the surrounding seawater (and over 1% in sample sp3). Whereas *Actinobacteria* and *Chloroflexi* were both common taxa in the individual hosts from Spain, these were rare in the present findings, which can be attributed to the high percentage of unassigned sequences. Two other common groups found in *S. plicata* from Spain were *Firmicutes* and *Cyanobacteria*, which were found to be rare in the current work, except for *Firmicutes* in sample sp3 (>2%). In addition, *Acidobacteria* and the candidate phyla OP3 were also found in Spain.

## Summary

The introduction of ascidians into new niches is facilitated by their ability to settle on docks, ropes, ship hulls, and other artificial substrates. Settling in a new location such as a harbor may serve as a starting point for a species to become invasive and establish its population in the surrounding natural environment. As we have demonstrated here, *Styela plicata* is rapidly spreading in marinas and fishing harbors along the Mediterranean coast of Israel. We suggest that the main factor responsible for its abundance, which may have influenced its establishment, is



**Fig. 2:** Taxonomic composition of three *S. plicata* individuals and three seawater samples (shown as an average), based on a 97% cut-off sequence similarity to known OTUs. Seawater samples revealed no significant difference among the samples. Phyla-level groups are shown for bacteria; *Proteobacteria* are represented at the Class level.

the harbor's level of maintenance (i.e. pulling out unused ropes, cleaning the dock pillars, and removing sessile organisms), the number of ropes in the water, and the type of boating activity. Consequently, the old fishing harbors of Jaffa and Akko are rich in *S. plicata*, and so too is Ashqelon, due to its active fishing wharf and the relatively high number of suspended ropes in the water. In contrast, well-maintained marinas (mostly type B), with fewer artificial constructions (e.g. cages and traps) or old ropes suspended in the water, were found to feature significantly lower numbers of *S. plicata*. We strongly recommend implementing the above-noted maintenance procedures as a standard policy. Further surveys of such artificial substrates should assist in monitoring the arrival and spread of this and other non-indigenous ascidian species.

The diverse microbial communities found in *S. plicata* differed from those of the surrounding water, indicating that at least some of the symbionts associated with this ascidian species are not a transient community. This is in accordance with Erwin *et al.* (2013) findings for the same species collected in Spain. Although we used the same number of animal samples as those in the study from Spain, we found a much higher diversity among our three samples. It is interesting, however, that the same bacterial phyla exist in *S. plicata* samples from different parts of the world. These associations hint at the role played by

these communities in helping their host to adapt to a new environment. Bacterial symbionts, for example, may assist their host to adapt to different surrounding water conditions, which is of particular importance for *S. plicata* in light of its global distribution (Pineda *et al.*, 2011) and the patterns we have shown here of its establishment. In addition, some bacterial phyla observed in host individuals are found in metal-polluted environments (OTU 0006; Table S2; Fidalgo *et al.*, 2016), or in anaerobic conditions (OTU 0001; *Chloroflexi*; Table S2); OTU 0001, similar to OTU 0057 (*Proteobacteria* associated with an invasive sponge; Table S2), being sponge-associated (Taylor *et al.*, 2007). Phyla found in *S. plicata* that are absent from seawater samples, may have been acquired through vertical transmission, although no bacteria were observed in transmission electron micrographs of the animals' gonads (Erwin *et al.*, 2013). Alternatively, through horizontal transmission, *S. plicata* may select for particular bacterial phyla present in the seawater at a given time and preserve these taxa in its tunic for the long term. This latter mechanism may also explain the high bacterial diversity found in the animal samples. Further research on this topic, comparing *S. plicata* across different seasons and sites, may shed light both on the microbial community structure and on the way in which these non-indigenous ascidians cope with pollution in harbors and under new environmental

conditions in general, as well as on the overall contribution of microbial symbionts to the ascidians' successful establishment in a new area.

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