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RESEARCH ARTICLE

A comparison of prokaryotic symbiont communities in nonnative and native ascidians from reef and harbor habitats

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One sentence summary: Sea-squirts possess prokaryotic symbionts that allow them to thrive in a wide range of habitats.

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ABSTRACT

Harbor systems represent passive gateways for the introduction of nonnative ascidians that compete with the surrounding benthos and may spread through localized dispersal, even populating adjacent natural reefs. To investigate the potential role of microbial symbionts in the success of ascidian introductions and spread, we evaluated the host-specificity of prokaryotic communities within two ascidian species commonly found off the North Carolina coast. Replicate samples of the native ascidian *Eudistoma capsulatum*, the nonnative ascidian *Distaplia bermudensis* and seawater were collected from artificial (harbor) and natural reef substrates. Prokaryotic communities in seawater samples and ascidian tunics were characterized via next-generation sequencing of partial 16S rRNA gene sequences. Ascidian microbiomes clustered strongly in response to host species, with significant differences in community structure between the two species and seawater. Further, symbiont community structure differed significantly between *E. capsulatum* individuals collected from artificial and natural habitats, though this was not the case for *D. bermudensis*. These findings suggested that some ascidian species possess stable microbial symbiont communities that allow them to thrive in a wide range of habitats, while other species rely on the restructuring of their microbial communities with specific symbionts (e.g. *Chelativorans*) to survive under particular environmental conditions such as increased pollution.

Keywords: tunicate; sea-squirt; symbiosis; introduced species; microbiome; 16S rRNA

INTRODUCTION

Biological invasions represent one of the greatest threats to biodiversity today (Doherty *et al.* 2016), with profound ecological consequences and long-term effects that are not yet well understood. Once established in a new region, nonnative species are often extremely difficult, if not impossible, to control (Simberloff, Schmitz and Brown 1997), as they can benefit from reduced predation (Mumby, Harborne and Brumbaugh 2011), as well as fewer pathogenic (Mitchell and Power 2003) and parasitic

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pressures (Torchin *et al.* 2003). Marine biological invasions are especially challenging to prevent, as few physical barriers exist to impede nonnative species entry. Though active (intentional) biological introductions are responsible for the introductions of some nonnative species into marine systems, passive (unintentional) introductions have likely occurred in the marine environment since the first shipping routes were established (Lambert and Lambert 2003). With the ever-increasing globalization of modern society, the rate of these unintentional marine biological introductions will continue to increase (Cohen and Carlton 1998), unless preventative measures are developed and implemented.

Ascidians are highly diverse members of the phylum Chordata, with approximately 3000 described species (Shenkar and Swalla 2011), and some of the highest recorded numbers of introduced species (Lambert and Lambert 2003). Ascidian larvae are lecithotrophic, short-lived and capable of only limited dispersal (Olson 1985), and adults are completely sedentary. Yet many ascidian species have been documented thousands of kilometers from their native range (Lambert and Lambert 1998; Shenkar and Swalla 2011; Zhan *et al.* 2015). This unnatural dispersal indicates that human-mediated transport is almost certainly responsible for most ascidian introductions (Lambert and Lambert 1998), which makes ascidians an ideal taxon to assess marine bioinvasions (Lambert and Lambert 1998; Zhan *et al.* 2015). Other taxa often surveyed for new marine introduced species include algae, hydrozoans, bryozoans and molluscs (Ruiz *et al.* 1997; Inderjit, Ranelletti and Kaushik 2006; Megina, González-Duarte and López-González 2016). As for the other taxa, the presence of ascidians attached to ship hulls outside their native range is well-documented (Lambert 2002; Gewing and Shenkar 2017), and live ascidian larvae have occasionally been detected within ballast water (Carlton and Geller 1993). Consequently, introduced ascidian species are typically first observed in harbor environments characterized by high shipping and recreational boating activities (Marins *et al.* 2010; López-Legentil *et al.* 2015a), and several species now exhibit a worldwide spread (Lambert and Lambert 1998; Shenkar and Swalla 2011; Zhan *et al.* 2015). All colonial ascidians and several solitary species are capable of internally brooding larvae (Bishop and Sommerfeldt 1996; Lambert 2002), and many are further characterized by high growth and fecundity rates (Grave 1933), such that just a few adult individuals are capable of establishing a robust founder population within a relatively short span of time (Lambert and Lambert 1998).

Some degree of adaptability to new environmental conditions is critical for any species to be successful in the long-term (Devin and Beisel 2006), but ascidians represent a particularly adaptable lineage, exhibiting a high tolerance to fluctuations in temperature (Nomaguchi *et al.* 1997; Lambert and Lambert 2003; Pineda *et al.* 2012; Nagar and Shenkar 2016), salinity (Sims 1984; Pineda *et al.* 2012; Nagar and Shenkar 2016) and pollution (Lambert 2007; Pineda *et al.* 2012; Stabili *et al.* 2015). As harbor environments are typically more shallow, enclosed and exhibit lower water quality compared to nearby natural reefs (Marins *et al.* 2010), harbor-based ascidians will experience greater fluctuations of environmental parameters and higher concentrations of organic and inorganic pollutants (McGee *et al.* 1995; Schiff *et al.* 2004; Renzi *et al.* 2009; Marins *et al.* 2010; Newton *et al.* 2011) compared to their deeper, more isolated reef counterparts. In benthic communities, increased pollution has been linked to shifts in dominance from native to introduced species, including ascidians (Piola and Johnston 2008). Further, harbors feature abundant, unoccupied surfaces available for colonization, typically artificial structures such as ropes and pilings (Lambert 2007), which frequently become dominated by populations of nonnative ascidians (Lambert and Lambert 1998; Marins *et al.* 2010). Following the establishment of a founder population, nonnative ascidians can spread progressively onto natural substrate (Bullard *et al.* 2007; Lambert 2007), outcompeting native species through physical overgrowth (Nandakumar, Tanaka and Kikuchi 1993; Lambert 2007) or the production of secondary metabolites to inhibit competitor growth or larval recruitment (Jackson and Buss 1975; Lambert 2005). Indeed, long-term surveys of harbor pilings have witnessed the introduction of numerous nonnative ascidian species that have persisted and increased in abundance, with previously established native ascidian populations experiencing corresponding declines (Lambert and Lambert 2003).

With the recent advent of next-generation sequencing, a growing body of evidence has revealed that ascidians harbor highly diverse and host-specific communities of prokaryotic symbionts within their inner tunic (Erwin et al. 2013, 2014; Evans *et al.* 2017). The term 'symbiosis' is used here following its original definition (*sensu* de Bary 1879) as the living together of different organisms, with no indication on the outcomes of these interactions (i.e. mutualistic, parasitic, and commensal relationships). Ascidians are also capable of maintaining similar symbiont community structure across broad spatial (Cahill *et al.* 2016) and temporal scales (Martínez-García *et al.* 2007; López-Legentil *et al.* 2015b). Further investigation has suggested that at least some of these host-symbiont relationships may be mutualistic (Maruyama, Hirose and Ishikura 2003; Hirose and Maruyama 2004), and that some symbiont capabilities (e.g. nitrification) may translate to host functionality (Hirose and Maruyama 2004; Martínez-García *et al.* 2007). The presence of particular symbiont taxa could therefore represent a competitive advantage for a nonnative ascidian entering a new region (Evans *et al.* 2017). However, only a few studies have examined the microbial symbionts of nonnative ascidians, revealing intraspecific variability or stability depending on the host species (Erwin *et al.* 2013; Cahill *et al.* 2016; Novak *et al.* 2017) and a high degree of host-specificity (Evans *et al.* 2017). The introduction of even a single ascidian individual outside of its native range also represents the simultaneous introduction of a complex community of interacting microorganisms, which makes understanding and controlling these biological invasions much more difficult. Moreover, the presence or absence of specific symbionts has been directly implicated in the ability of some invasive species to successfully establish founder populations within a new region (Richardson *et al.* 2000; Parker, Malek and Parker 2006; Simonsen *et al.* 2017). Thus, an understanding of the functional role of microbial symbionts in nonnative species may be critical to bioinvasion prevention and management.

Although some marine invertebrates have been shown to maintain similar symbiotic microbial communities across broad geographic scales (sponges: Hentschel *et al.* 2002; Pita *et al.* 2013; ascidians: Cahill *et al.* 2016; oysters: Pierce 2016), others possess more fluid microbial communities that shift in response to changes in the environment (corals: Littman *et al.* 2009; sponges: Burgsdorf *et al.* 2014). Similarly, for introduced organisms, the presence or absence of specific symbionts has been shown to be limiting for some species (Parker, Malek and Parker 2006; Rout *et al.* 2013; Simonsen *et al.* 2017) but not others (Lee and Hooper-Bui 2012; Coats 2013; Bansal, Mian and Michel 2014; Eichmiller *et al.* 2016). Much of this variation could be the result of differences in symbiont acquisition strategies, whether vertical (passed from parent to offspring) or horizontal (sourced from

the external environment). Vertical transmission would ensure that offspring possess critical symbionts, but could limit adaptability to new environments. Horizontal acquisition could allow for faster acclimation to a new habitat, but could risk the loss of critical symbionts. Importantly, these two acquisition strategies are not mutually exclusive; mixed strategies utilizing both forms of symbiont acquisition (i.e. 'leaky vertical transmission,' Vrijenhoek 2010) could prove especially beneficial for introduced species.

Within harbors, high pollution levels can result in dramatic, permanent changes to the structure of the ascidian community (Goodbody 1993; Turner *et al.* 1997), leading to shifts in dominance and the success of introduced ascidians at the expense of native populations (Lambert and Lambert 2003; Piola and Johnston 2008). Pollution can also impact the makeup of marine bacterioplankton communities (Zhang *et al.* 2009), but how poor water quality may impact the symbiotic association between ascidians and their microbes is unclear. Here, we aimed to characterize the prokaryotic symbiont communities of a native and a nonnative ascidian species collected from both artificial (harbor) and natural reef environments off the North Carolina coast. Our goal was to determine whether microbial symbionts could play a role in the successful establishment and spread of ascidians in these two different habitats.

METHODS

Sample collection, processing and DNA extraction

Two colonial ascidian species within the order Aplousobranchia were examined in this study: the nonnative *Distaplia bermudensis* (van Name 1902), and the native *Eudistoma capsulatum* (van Name 1902). *D. bermudensis* was first described in Bermuda (van Name 1902), but exhibits a worldwide distribution, with documented sightings in Puerto Rico (van Name 1945), the southeastern US (van Name 1945; Villalobos *et al.* 2017), Brazil (Rocha and Kremer 2005), Spain (Peres 1957) and Italy (Mastrototaro and Brunetti 2006). *E. capsulatum* is considered native to the southeastern US and the Caribbean (van Name 1945; Villalobos *et al.* 2017) and to our knowledge has not been reported outside its native range.

In March 2016, replicates of each ascidian species ($n = 5$) and ambient seawater ($n = 3$) were collected at the Bridge Tender Marina (34°13′06″N; 77°48′47″W) in Wilmington (North Carolina; US) and at a natural reef site, 5-Mile Ledge (34°06′08″N; 77°45′03″W), located less than 15 km from the harbor site. Reef samples were collected via SCUBA at <17 m in depth, and harbor samples were collected by snorkeling at < 2 m depth. Isolated colonies were collected to minimize the potential of sampling clones. Samples were housed in ambient seawater and dissected in the laboratory (less than 10 km away from sampling sites). Ascidian samples were fixed in absolute ethanol and stored at −20°C. Prior to DNA extraction, each colony piece was further subdivided into zooid (formed by a thorax and an abdomen and thus rich in animal DNA) and inner tunic fractions (i.e. tunic tissue not in contact with the ambient seawater or the zooids and where most of the symbiotic prokaryotes are observed; Erwin *et al.* 2014) under a stereomicroscope for use in host-barcoding and symbiont characterization, respectively. Triplicate ambient seawater samples from each site (500-ml each) were concentrated onto 0.2-μm filters and immediately frozen at −80°C. DNA extractions of zooids, tunic samples, and seawater filters were performed with the DNeasy Blood and Tissue Kit (Qiagen).

Genetic barcoding of ascidian hosts

Ascidian zooids were barcoded by sequencing a ca. 600-bp segment of the mitochondrial COI gene using either the 'universal' LCO1490 and HCO2198 primers (Folmer *et al.* 1994) or the ascidian-specific Tun_forward and Tun_reverse2 primers (Stefaniak *et al.* 2009). PCR amplifications were performed with a total volume of 25 μl, consisting of 5 pmol of each primer, 2X MyTaq HS Red Mix (Bioline) and 1 μl (ca. 10 ng) of template DNA in an Eppendorf Mastercycler nexus gradient. The thermocycler program included an initial denaturation at 95°C for 1 min; 35 cycles of 95°C for 15 s, 45°C for 15 s and 72°C for 10 s; and a final extension at 72°C for 1 min. All sequence reactions were conducted with BigDye Terminator v. 3.1 (Applied Biosystems) and the same forward and reverse primers utilized in the initial amplifications. Polymerase chain reaction (PCR) products were then purified using BigDye XTerminator (Applied Biosystems) and sequenced on an AB 3500 genetic analyzer (Applied Biosystems) located at the UNCW Center for Marine Science. Raw sequence reads were processed in Geneious version 8.02 (Kearse *et al.* 2012) through the alignment of forward and reverse reads into consensus sequences. These consensus sequences were compared to the GenBank database using nucleotide-nucleotide BLAST searches (BLASTn) to confirm host identity based on the highest % match (Altschul *et al.* 1990; Table S1, Supporting Information), and archived in GenBank with the accession numbers MG525006 to MG525023.

Sequencing of prokaryotic symbionts

To characterize the prokaryote communities in ascidian tunics and seawater, a ca. 300 bp fragment (V4 region) of the 16S ribosomal RNA (rRNA) gene was amplified and sequenced using the universal bacterial/archaeal forward primer 515f and reverse primer 806r (Caporaso *et al.* 2011). DNA extractions were initially tested for viability through PCR amplification in an Eppendorf Mastercycler nexus gradient, with a thermocycler program consisting of an initial denaturation at 95°C for 2 min; 35 cycles of 95°C for 15 s, 50°C for 15 s and 72°C for 20 s; and a final extension at 72°C for 2 min. PCR-viable DNA extracts were subsequently sent to Molecular Research LP for amplification, library construction and multiplexed sequencing of partial 16S rRNA gene sequences using the same 515f and 806r primers as above on an Illumina MiSeq platform. Raw sequences were deposited in the Sequence Read Archive of NCBI (accession no. SRP132224).

Processing of next-generation sequence data

Raw sequences were processed within the mothur software package (v.1.38.0; Schloss *et al.* 2009), following a modified version of the Illumina MiSeq SOP pipeline (Kozich *et al.* 2013). Briefly, raw sequences were quality-filtered and aligned with the SILVA reference database (v128; Pruesse *et al.* 2007), and putative chimeric sequences were removed through self-reference searches using UChime (Edgar *et al.* 2011). Sequences were classified on the basis of a naive Bayesian classifier and bootstrap algorithm for confidence scoring (Wang *et al.* 2007), based on the improved Greengenes taxonomy (McDonald *et al.* 2012), and nontarget sequences (chloroplasts, mitochondria, and eukaryotes) and singletons were removed from the data set. High-quality sequences were assigned to operational taxonomic units (OTUs) in mothur based on 97% sequence similarity and the opticlust clustering algorithm, and the taxonomic classification of each OTU was determined by majority consensus (Schloss and

Westcott 2011). Each data set was subsampled to the lowest read count ($n = 31952$) from the final shared file in order to standardize sampling depths (i.e. number of sequence reads) among the different replicates, and all subsequent data analyses were based on these subsampled data sets.

One specimen of *E. capsulatum* from the reef site and one from the harbor exhibited disproportionately lower numbers of reads ($n = 12669$ and $n = 16050$, respectively) and were therefore removed from the analysis through subsampling. To confirm that the detected significant differences in microbial community structure (see below) were not the result of the removal of these two samples, all subsequent analyses were repeated based on a subsampling depth of $n = 12669$ to include all replicates. Equivalent results were observed between host types, locations and interactive effects between the two variables (Table S2 and Figure S1, Supporting Information). Consequently, these two samples were removed from the final analysis to allow for a greater sampling depth across all remaining samples.

Analysis of symbiont community diversity

Alpha diversity metrics OTU richness, evenness and diversity were calculated in mothur to compare prokaryotic community diversity among the six sources (two ascidian species and seawater at two sites). Metrics utilized included the OTU richness indices S (total number of OTUs observed) and Chao 1 (expected number of OTUs), and the Simpson evenness index (E1/D), the inverse Simpson index (D) and the Shannon–Weaver diversity index (H'). Analyses of variance (ANOVA) were used to statistically compare the diversity indices for the factors source (*D. bermudensis*, *E. capsulatum*, and seawater), site (harbor *vs*. reef) and an interaction term, with Tukey's honest significance difference (HSD) tests performed for multiple pairwise post hoc comparisons of means.

Analysis of symbiont community structure

Beta diversity metrics for OTU overlap and similarity of prokaryotic symbiont communities were calculated to compare host-specificity and community structure differences between the six sources. Venn diagrams were constructed in mothur to visualize OTU overlap among sources. Prokaryotic communities were compared via Bray–Curtis similarity (BCS) matrices based on OTU relative abundances and visualized in non-metric multidimensional scaling plots using PRIMER (v7.0.13; Clarke and Gorley 2015). Permutational multivariate analyses of variance (PERMANOVA) were used to compare the structure of prokaryotic communities for the factors source (*D. bermudensis*, *E. capsulatum* and seawater), site (harbor *vs*. reef) and an interaction term, with significance determined using permutational *p*-values or Monte Carlo asymptotic *p*-values in comparisons with few permutations (i.e. pairwise comparisons involving seawater). Multiple pairwise comparisons were performed for significant main test PERMANOVA results and were corrected via the Benjamini–Yekutieli (B–Y) false-discovery rate control and an experiment-wise error rate of $\alpha = 0.05$ (Benjamini and Yekutieli 2001). All significant PERMANOVA results were further analyzed with permutational multivariate analyses of dispersion (PERMDISP) to verify that all significant PERMANOVA results represented actual structural differences, rather than unequal variability of dispersion among the six sources. To determine whether significant differences in community structure occurred among rarer OTUs, sequence data were divided into abundant and rare-OTU data partitions, with a 0.1% cutoff threshold (Fuhrman 2009). This resulted in a cutoff value of 32 sequences, with OTUs containing > 32 sequences considered 'abundant,' and OTUs containing ≤ 32 sequences considered 'rare.' BCS matrices were created for each data partition and tested for differences in the community structure (PERMANOVA) of abundant and rare taxa, as described above. Similarity percentage (SIMPER) analyses were further utilized to identify the top OTUs responsible for the dissimilarity between symbiont communities from different sources. As SIMPER analyses may confound variability between and within groups (Warton, Wright and Wang 2012), an additional statistical test (Metastats, White, Nagarajan and Pop 2009) was conducted in mothur to determine which SIMPER OTUs exhibit significantly different relative abundances among groups based on 1000 permutations.

RESULTS

Symbiont community composition

In all, 766848 sequences, consisting of 6010 distinct OTUs, were obtained from the six sources (two ascidian species and seawater samples from two sites; total $n = 18$ ascidian samples and $n = 6$ seawater samples). Seawater bacterioplankton communities represented 4002 OTUs ($n_{reef} = 2632$ and $n_{harbor} = 2419$), with just 1049 of these present in the seawater at both locations (Figure S2, Supporting Information). Ascidian symbiont communities represented 3877 OTUs, with 2008 of these unique to ascidian hosts and not represented within the bacterioplankton community (Figure S3, Supporting Information). For interspecific comparisons of ascidian symbiont communities, *Distaplia bermudensis* contained 1008 unique OTUs (total $n = 2205$), while *Eudistoma capsulatum* hosted 1672 unique OTUs (total $n = 2869$), with 1197 OTUs shared between the two host species (Fig. 1). For intraspecific comparisons of ascidian symbiont communities with respect to site differences, *D. bermudensis* ($n_{reef} = 1605$ and $n_{harbor} = 1180$) possessed 1025 and 600 OTUs unique to the reef and harbor sites, respectively, and 580 OTUs were present in this species at both sites (Fig. 1). *E. capsulatum* ($n_{reef} = 1679$ and $n_{harbor} = 2038$) contained 831 and 1190 OTUs unique to the reef and harbor sites, respectively, with 848 OTUs detected in this species at both sites (Fig. 1).

The seawater bacterioplankton community spanned 40 bacterial and three archaeal phyla (*Euryarchaeota*, *Crenarchaeota* and *Parvarchaeota*; Fig. 2a). Ascidian symbiont OTUs spanned 39 bacterial and the same three archaeal phyla. Archaeal symbionts represented just 0.39% ($n = 2246$) of all ascidian prokaryotic sequences ($n = 575136$), with the majority of these archaeal reads (89%; $n = 2009$) identified as *Crenarchaeota* within just two ascidian samples: one *D. bermudensis* specimen from the reef, and one *E. capsulatum* specimen from the harbor (Figs 2b and e, respectively). Bacterial phyla likewise differed in relative abundances among the four ascidian sources. *D. bermudensis* symbiont communities of reef origin included 29 bacterial phyla but were dominated by *Alphaproteobacteria* (52%), *Gammaproteobacteria* (12%) and *Bacteroidetes* (10%), as well as bacteria unclassified below the kingdom level (23%; Fig. 2b). The majority of these unclassified bacterial sequences (94.9%) consisted of a single OTU (OTU00003). *E. capsulatum* from the reef possessed symbiont communities spanning 32 bacterial phyla, predominantly *Alphaproteobacteria* (70%), *Gammaproteobacteria* (11%), SBR1093 (7%) and *Bacteroidetes* (7%; Fig. 2c). Within the harbor environment, *D. bermudensis* symbiont communities were comprised of 25 bacterial phyla, predominantly *Alphaproteobacteria* (39%),

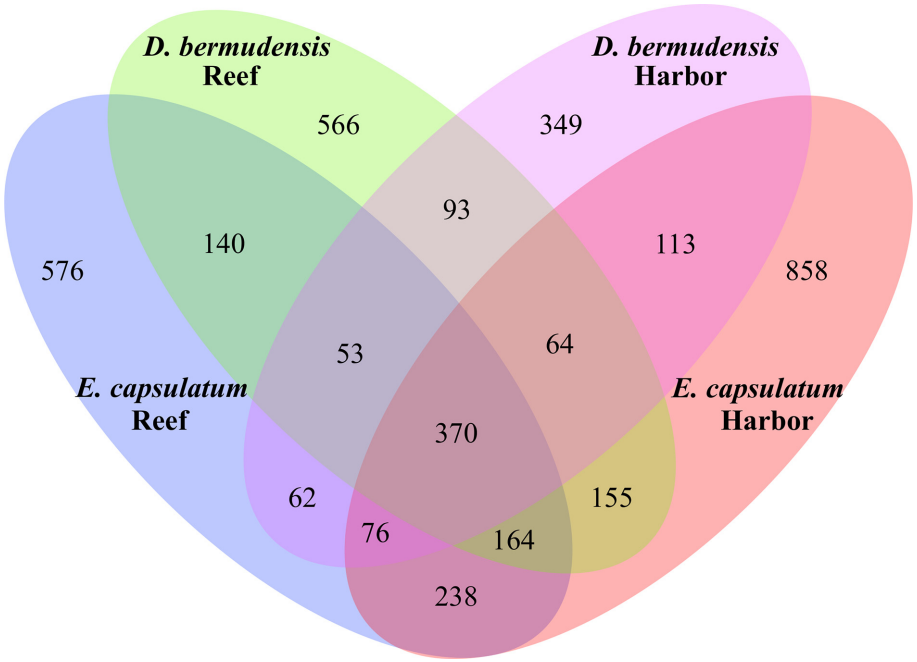

Figure 1. Venn diagram showing OTU overlap in prokaryotic symbiont communities of *D. bermudensis* and *E. capsulatum* collected from harbor and natural reef environments. *D. bermudensis* is depicted in pink and green for the harbor and reef, respectively. *E. capsulatum* is shown in red and blue for the harbor and reef, respectively. Total OTU richness was 3877 OTUs among the four ascidian sources.

Gammaproteobacteria (33%) and *Bacteroidetes* (6%), but also unclassified bacteria (20%; Fig. 2d). Similar to the reef site, OTU000003 represented the vast majority (88.5%) of these unclassified bacterial sequences. *E. capsulatum* from the harbor contained symbionts from 35 bacterial phyla, with the majority of symbionts classified as *Alphaproteobacteria* (55%), *Gammaproteobacteria* (27%), *Bacteroidetes* (5%; Fig. 2e), *Betaproteobacteria* (4%) and *Nitrospirae* (3%).

Symbiont community structure

Prokaryotic communities clustered in response to source (Fig. 3). Significant differences were detected between the symbiont communities of the two ascidian hosts (*D. bermudensis* and *E. capsulatum*) and ambient seawater (PERMANOVA, $P = 0.001$), which explained 49.9% of the observed community structure variation. All subsequent pairwise comparisons among host species and seawater were likewise significant (PERMANOVA, $P = 0.001$). Within each source type, seawater exhibited less variability in prokaryotic community structure among replicates (average similarity = 66.6%) compared to the microbiomes of either ascidian, and *D. bermudensis* (average similarity = 41.2%) exhibited greater similarity among replicates than *E. capsulatum* (average similarity = 29.5%). Accordingly, the permutational multivariate analysis of dispersion across groups revealed significant differences among the three source types (PERMDISP, $P = 0.004$). However, subsequent pairwise comparisons of the six sources revealed that all significant differences in dispersion (PERMDISP, $P < 0.05$) occurred in comparisons of ascidian symbiont communities to seawater bacterioplankton communities, and none of these remained significant following B–Y correction (Table 1). All pairwise comparisons between ascidian hosts revealed no significant differences in dispersion (Table 1). A significant interactive effect between host and site was also detected (PERMANOVA, $P = 0.035$) and explained an additional 8.47% of the observed variation in prokaryotic community structure.

Within each site, interspecific pairwise comparisons revealed significant differences between both ascidian species, as well as between both ascidian species and ambient seawater (Table 1; Table S3, Supporting Information). Between the two sites, intraspecific pairwise comparisons revealed significantly different symbiont community structure for *E. capsulatum* from the reef compared to the harbor ($P = 0.030$), though this difference was not significant following B–Y correction (Table 1). Inclusion of all *E. capsulatum* replicates (see Methods) increased the power of this comparison, resulting in a significant outcome robust to B–Y correction ($P = 0.013$, Table S2, Supporting Information). For *D. bermudensis*, pairwise comparisons revealed no significant differences in symbiont community structure between the two sites ($P = 0.106$, Table 1). Additionally, greater similarity was observed between prokaryotic communities of *D. bermudensis*, regardless of site, than between corresponding communities within *E. capsulatum* (41.2 and 29.5% average similarity, respectively). Significant structural differences were also detected between the seawater bacterioplankton communities of the harbor compared to the reef ($P = 0.002$; Table 1).

In order to determine the effects of OTU relative abundance on observed community structure differences, symbiont community data were partitioned into abundant (> .1% relative abundance) and rare (≤ 0.1%) components. Partitioning resulted in 414 abundant OTUs that accounted for 97.5% of all sequences and 5596 rare OTUs that accounted for the remaining 2.5%, following singleton removal. Both the abundant and rare data partitions indicated significant differences among sources (PERMANOVA, $P = 0.001$ for both partitions) and sites (PERMANOVA, $P = 0.004$ and $P = 0.006$, respectively), as well as a significant interactive effect between the two variables (PERMANOVA, $P = 0.036$ and $P = 0.001$, respectively). Interspecific pairwise comparisons revealed the same trends for the abundant data partition as the overall partition; however, pairwise

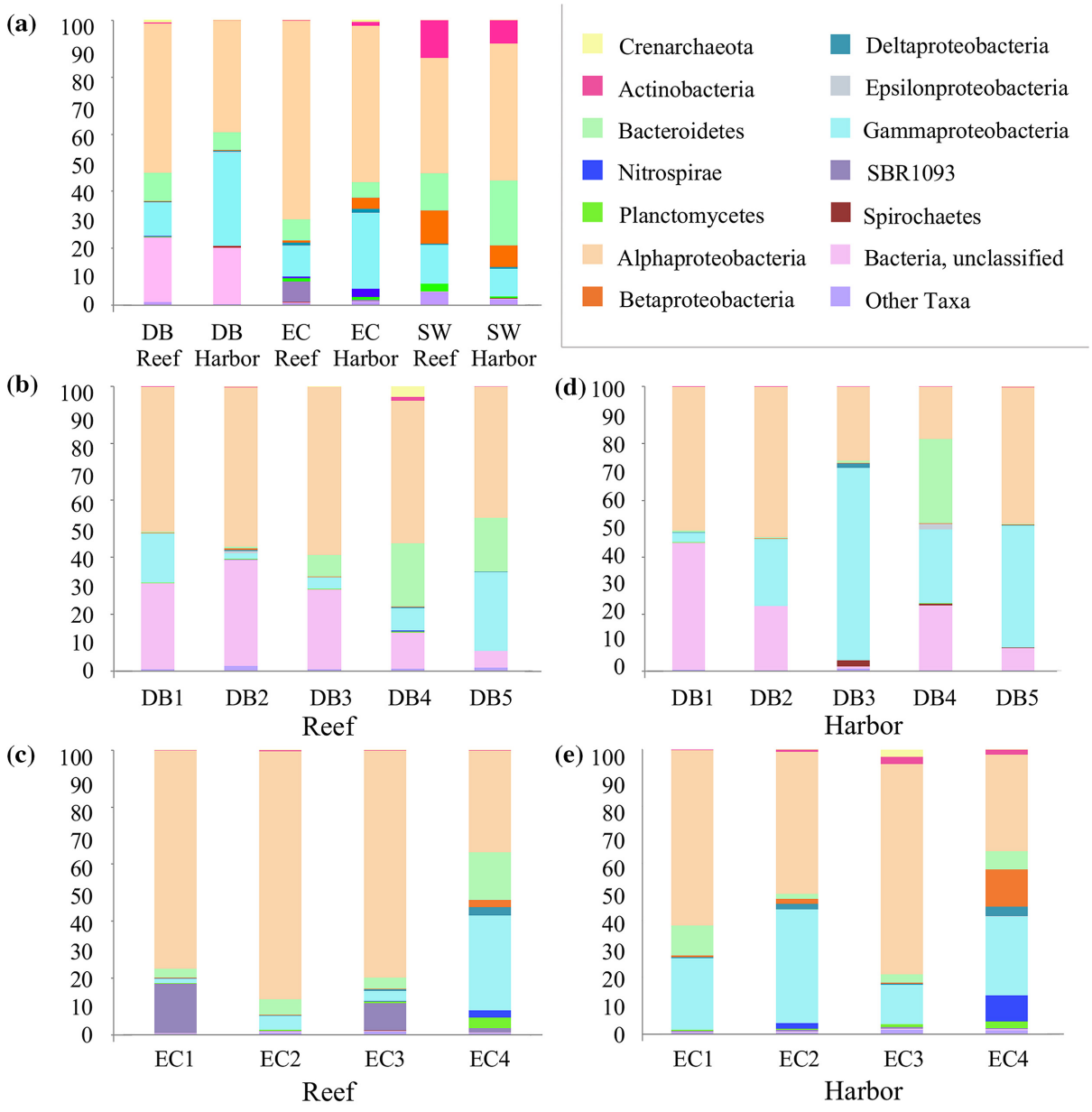

Figure 2. Symbiont community composition averaged for the four ascidian sources and the seawater (a), and for each replicate sample of each ascidian source: *D. bermudensis* (b) and *E. capsulatum* (c) from the reef, and *D. bermudensis* (d) and *E. capsulatum* (e) from the harbor. Phylum-level classifications are shown except for Proteobacteria, which are divided into their five major classes. Other taxa = *AC1*, *Acidobacteria*, *AD3*, *AncK6*, *Armatimonadetes*, *Chlamydiae*, *Chlorobi*, *Chloroflexi*, *Cyanobacteria*, *Elusimicrobia*, *Euryarchaeota*, *Fibrobacteres*, *Firmicutes*, *Fusobacteria*, *Gemmatimonadetes*, *GN02*, *LD1*, *Lentisphaerae*, *NKB19*, *OD1*, *OP11*, *OP3*, *OP8*, *Parvarchaeota*, *PAUC34f*, *SAR406*, *SR1*, *TA18*, *Tenericutes*, *Thermi*, *TM6*, *TM7*, unclassified *Archaea* and *Proteobacteria*, *Verrucomicrobia*, *WPS-2*, *WS3*, *WS5*, *ZB3* and *Zetaproteobacteria*.

comparisons between ascidian hosts and seawater were not significant for the rare data partition (Table 1). For the abundant data partition, intraspecific comparisons between the reef and harbor revealed no significant difference in symbiont community structure of *D. bermudensis* at the two sites ($P = 0.133$), while a significant difference was detected between *E. capsulatum* symbiont communities at the two sites prior to B–Y corrections ($P = 0.029$, Table 1). For the rare data partition, significant differences were detected across sites for both *D. bermudensis* ($P = 0.032$) and *E. capsulatum* ($P = 0.029$) intraspecific symbiont community structure, but were not robust to false discovery rate corrections (Table 1). Significant differences in dispersion across sources were detected for both the abundant and rare data partitions (PERMDISP, $P = 0.006$ and $P = 0.001$, respectively). Subsequent pairwise comparisons revealed that only the seawater-ascidian comparisons exhibited significant differences in dispersion for the abundant data partition, and these did not remain significant following B–Y correction. Additional ascidian-ascidian comparisons were significant for the rare data partition (Table 1). Neither data partition revealed significant differences in dispersion within sources across the two sites (Table 1).

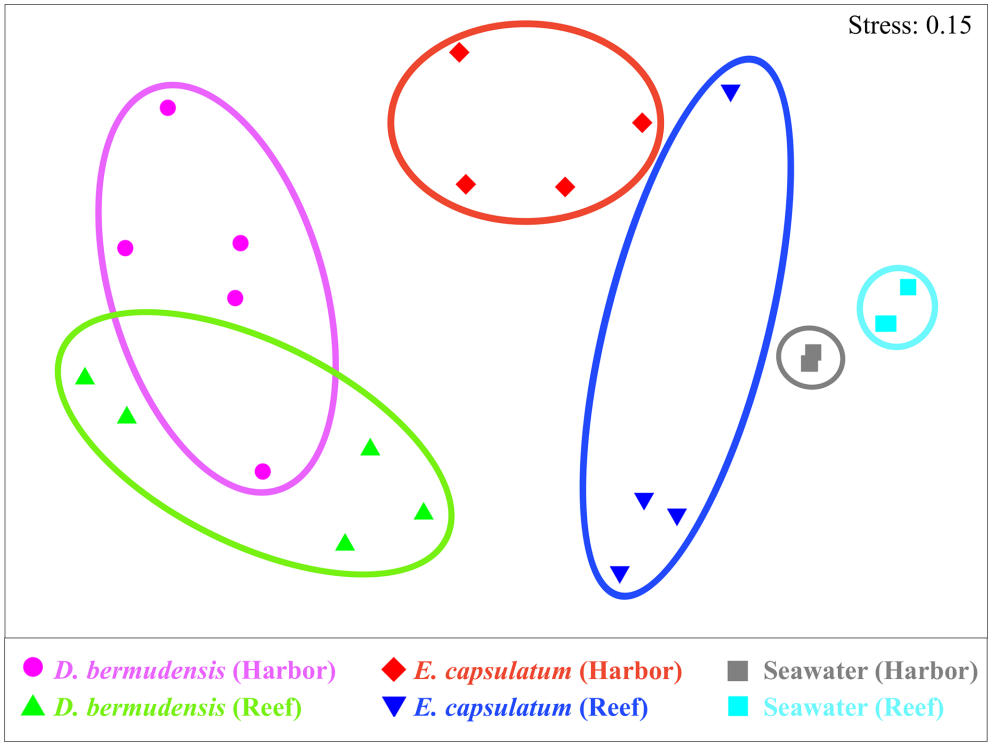

Figure 3. Non-metric multi-dimensional scaling plot based on BCS of prokaryotic communities in harbor-sourced *D. bermudensis* (pink circles), *E. capsulatum* (red diamonds) and seawater (gray squares), and reef-sourced *D. bermudensis* (green triangles), *E. capsulatum* (blue triangles) and seawater (aqua squares). Circles encompass replicate samples within each source and indicate a high degree of interspecific host-specificity.

Table 1. Pairwise statistical comparisons of microbial community structure (PERMANOVA) and dispersion (PERMDISP) in *D. bermudensis*, *E. capsulatum* and ambient seawater from reef and harbor sites at overall, abundant and rare data partition levels.

		Overall				Abundant				Rare			
		PERMANOVA		PERMDISP		PERMANOVA		PERMDISP		PERMANOVA		PERMDISP	
Site	Pairwise comparison	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*
Reef	*D. bermudensis*, *E. capsulatum*	2.08	0.012*	0.22	0.897	2.10	0.009*	0.23	0.905	1.09	0.015*	3.58	0.011*
	D. bermudensis, seawater	3.13	0.002*[m]	4.88	0.017	3.17	0.005*[m]	5.01	0.019	1.41	0.075 [m]	17.42	0.025
	E. capsulatum, seawater	2.84	0.008*[m]	4.27	0.060	2.89	0.007*[m]	4.36	0.048	1.42	0.118 [m]	13.58	0.025
Harbor	*D. bermudensis*, *E. capsulatum*	2.28	0.012*	1.73	0.206	2.30	0.009*	1.69	0.253	1.23	0.007*	3.83	0.009*
	D. bermudensis, seawater	3.94	0.001*[m]	4.32	0.035	4.00	0.001*[m]	4.49	0.030	1.39	0.091 [m]	19.24	0.016
	E. capsulatum, seawater	2.75	0.008*[m]	11.51	0.025	2.80	0.006*[m]	11.64	0.027	1.29	0.175 [m]	8.83	0.037
Reef & Harbor	*D. bermudensis*, *D. bermudensis*	1.54	0.106	1.16	0.251	1.55	0.133	1.14	0.251	1.07	0.032	0.03	0.961
	E. capsulatum, *E. capsulatum*	1.61	0.030	0.58	0.680	1.62	0.029	0.58	0.684	1.13	0.029	0.84	0.373
	Seawater, seawater	5.56	0.002*[m]	1.69	0.192	6.59	0.001*[m]	1.53	0.208	1.49	0.110 [m]	1.21	0.102

*significantly different following B–Y correction, [m] Monte Carlo *p*-value.

SIMPER analyses confirmed the high degree of dissimilarity between the two ascidian species within each site (Table 2). At the reef site, *D. bermudensis* and *E. capsulatum* symbiont communities exhibited 82%, 82% and 93% average dissimilarity for the overall, abundant and rare data partitions, respectively. Comparisons between the two species within the harbor site revealed an average dissimilarity of 82%, 82% and 95% for the overall, abundant and rare data partitions, respectively. SIMPER analyses also indicated that between the two sites, *D. bermudensis* symbiont communities were on average more similar than *E. capsulatum* symbiont communities. Comparing harbor-sourced ascidian prokaryotic communities to reef-sourced individuals, *D. bermudensis* symbiont communities exhibited 37%, 38% and 6% average similarity for the overall, abundant and rare data partitions, respectively, while *E. capsulatum* symbiont communities exhibited 24%, 24% and 8% average similarity between the two sites for the overall, abundant and rare data partitions, respectively. At the overall data partition level, the majority of observed dissimilarity between the four ascidian sources (> 50%) was driven by differences in the relative abundance of seven OTUs spanning five bacterial taxa: *Rhodobacteraceae*, *Endozoicimonaceae*, *Hyphomonadaceae*, *Novispirillum*, *Chelativorans* and an unclassified bacterial OTU (Table 2). Similarly, the majority of observed dissimilarity between the four ascidian sources in the abundant data partition was driven by a small number of OTUs (n = 3–4; Table S4, Supporting Information). In contrast, the majority of the dissimilarity (> 50%) observed between the rare symbiont communities in ascidians was driven by differences in the relative abundance of a large number of OTUs (n = 330 to 374; Table S5, Supporting Information).

Symbiotic community alpha diversity

To determine whether the overall differences in prokaryotic community structure between sources were also reflected within each source's prokaryotic community diversity, alpha diversity metrics were calculated by source and location using the overall data partition. Source type (two ascidian species and seawater) was found to have a significant effect (ANOVA, $P < 0.05$) on prokaryotic community diversity for all five alpha diversity indices. Pairwise comparisons indicated that seawater exhibited significantly greater diversity, evenness and richness than *D. bermudensis* for all five indices, and significantly greater diversity, evenness and richness than *E. capsulatum* for all but the Simpson evenness index (Table 3). *E. capsulatum* exhibited significantly greater diversity than *D. bermudensis* based on the S, Chao1 and H' indices (Table 3). Comparing the two sampled sites, location was found to have no significant effect on alpha diversity (Table 3). A significant interactive effect between the two variables source (two ascidian species and seawater) and location was detected only for the inverse Simpson index (D), for which the seawater at the two locations was found to be significantly different from one another (Table 3). For D, none of the interspecific pairwise comparisons revealed significant differences between the ascidians' symbiont community diversities (Table 3), and both ascidian species exhibited significantly lower prokaryotic diversity than the seawater at the reef, although only the nonnative ascidian species *D. bermudensis* exhibited significant differences from the bacterioplankton community diversity in the harbor (Table 3). None of the intraspecific comparisons between sites revealed significant differences in diversity, evenness or richness for any of the alpha diversity indices or species.

DISCUSSION

With global invasion rates on the rise (Cohen and Carlton 1998), a full understanding of the mechanisms contributing to invasive species establishment, survival and spread are critical to the development of successful management strategies. Harbors function as introduction gateways for nonnative ascidians, with founder populations typically first becoming established within harbors (Lambert and Lambert 1998; Lambert 2002, 2003; López-Legentil *et al.* 2015a) before subsequently spreading into surrounding natural reefs via localized natural dispersal (Lambert 2002, 2003; Turon, Nishikawa and Rius 2007). Here, we compared the prokaryotic symbiont communities of native and nonnative ascidian species across artificial (i.e. harbor) and natural reef environments. Our results revealed a high degree of host-specificity in the microbiomes of native and nonnative ascidian hosts and clear distinctions between ascidian-associated and ambient seawater communities. Further, we found that the microbiome of the native host *E. capsulatum* shifted across habitats, while the microbiome of the nonnative *D. bermudensis* remained stable, suggesting different eco-evolutionary constraints on symbiont communities in native and nonnative ascidians.

Ascidian microbiomes have been previously shown to be highly host-specific, with prokaryotic symbiont communities clustering in response to source, for both native (Erwin *et al.* 2014; López-Legentil *et al.* 2015b) and nonnative species (Cahill *et al.* 2016; Evans *et al.* 2017). We determined that *E. capsulatum* and *D. bermudensis* likewise hosted significantly different symbiont communities from one another, in terms of both structure and diversity, and within both artificial and natural habitats. Host-specificity was clearly evident among abundant prokaryotic symbionts, while rare symbiont communities exhibited greater variability among replicates within the same species and location. Additionally, both ascidian species at both sites hosted symbiont communities that were statistically different from those of the ambient seawater, thus confirming that the microbiomes of native and nonnative ascidians are not mere reflections of the external environment, as has also been reported for other ascidian species (Erwin *et al.* 2014; López-Legentil *et al.* 2015b; Cahill *et al.* 2016; Evans *et al.* 2017). In fact, over a third (33.4%) of all obtained sequences were detected within the ascidians alone and were not present in the seawater samples from either site.

This study represents the first intraspecific comparison of ascidian microbiomes across two different environments: an artificial harbor and a natural reef. Intraspecific comparisons among individuals of the nonnative host *D. bermudensis* collected from these two different environments revealed stable microbiomes with no significant differences due to location. Similar results have been reported for other marine invertebrates sampled from polluted harbors and adjacent natural reefs (Gantt, López-Legentil and Erwin 2017). In contrast, the microbiome of the native host *E. capsulatum* varied significantly between the two sites. The observed differences in symbiont community stability between ascidian hosts may result from variable symbiont acquisition strategies, environmental tolerances or a combination of both factors. In general, the degree to which ascidian symbionts are sourced vertically from the parent ascidian or horizontally from the external environment is unclear, with some evidence for both means of transmission. In addition to the high degree of host-specificity exhibited by the microbial communities of colonial ascidians, these animals also brood their larvae internally and bacteria have been observed within

Table 2. Taxonomic classification and relative abundance of symbiont OTUs contributing to the observed dissimilarity in microbial community structure (50% cumulative, SIMPER analyses) between *D. bermudensis* (DB) and *E. capsulatum* (EC) within the reef and harbor, for the overall data partition. Asterisks (∗) denote significantly different (Metastats, $P < 0.05$) OTU abundances between sources.

					Rel. Abund. (%)		Contribution to
Comparison	Dissimilarity (%)	OTU ID #	Phylum	Lowest Taxonomy†	DB Reef	EC Reef	Dissimilarity (%)
DB Reef, EC Reef	82.19	2	*Proteobacteria*	F. *Rhodobacteraceae*	28.0	21.0	13.72
		3	unclassified	K. *Bacteria*	21.7	0.2	13.05*
		1	*Proteobacteria*	G. *Novispirillum*	21.3	0.4	12.73
		6	*Proteobacteria*	F. *Rhodobacteraceae*	0.5	19.0	11.28*
					DB Harbor	EC Harbor	
DB Harbor, EC Harbor	81.97	1	*Proteobacteria*	G. *Novispirillum*	30.0	0.3	18.13*
		9	*Proteobacteria*	G. *Chelativorans*	0.1	26.1	15.87
		4	*Proteobacteria*	F. *Endozoicimonaceae*	30.7	16.6	12.97
		3	unclassified	K. *Bacteria*	17.5	0.19	10.57*
					DB Reef	DB Harbor	
DB Reef, DB Harbor	62.64	4	*Proteobacteria*	F. *Endozoicimonaceae*	3.6	30.7	22.34*
		2	*Proteobacteria*	F. *Rhodobacteraceae*	28.0	6.1	20.59*
		1	*Proteobacteria*	G. *Novispirillum*	21.3	30.0	20.09
					EC Reef	EC Harbor	
EC Reef, EC Harbor	76.04	9	*Proteobacteria*	G. *Chelativorans*	0.2	26.1	17.03
		2	*Proteobacteria*	F. *Rhodobacteraceae*	21.0	2.6	12.44*
		4	*Proteobacteria*	F. *Endozoicimonaceae*	0.4	16.6	10.65*
		6	*Proteobacteria*	F. *Rhodobacteraceae*	19.0	10.9	9.38
		11	*Proteobacteria*	F. *Hyphomonadaceae*	9.0	5.0	5.23

†K kingdom, P phylum, C class, O order, F family, G genus, S species.

Table 3. Diversity metrics for microbial communities associated with *D. bermudensis* and *E. capsulatum* from the harbor and reef sites. S: observed richness, Chao 1: expected richness, E1/D: Simpson evenness, D: inverse Simpson evenness and H': Shannon Weaver index. Average values (±1 SD) are shown, with different superscript letters denoting significantly different means among sources.

Source	S	Chao 1	E1/D	D	H'
D. bermudensis	461.0 ± 144.3^{C}	1052.6 ± 300.6^{C}	0.008 ± 0.002^{B}	3.345 ± 0.881^{B}	1.698 ± 0.328^{C}
E. capsulatum	737.5 ± 129.4^{B}	1441.7 ± 230.6^{B}	0.010 ± 0.005^{AB}	7.127 ± 4.694^{B}	2.702 ± 0.512^{B}
Seawater	1264.5 ± 134.2^{A}	2796.1 ± 227.4^{A}	0.013 ± 0.004^{A}	17.13 ± 7.006^{A}	3.864 ± 0.367^{A}
Location	S	Chao 1	E1/D	D	H'
Reef	767.4 ± 372.2^{A}	1642.6 ± 786.3^{A}	0.011 ± 0.005^{A}	9.444 ± 8.710^{A}	2.670 ± 1.042^{A}
Harbor	740.7 ± 342.6^{A}	1593.7 ± 762.8^{A}	0.009 ± 0.004^{A}	6.659 ± 4.751^{A}	2.478 ± 0.910^{A}
Source∗Location	S	Chao 1	E1/D	D	H'
D. bermudensis (reef)	522.0 ± 184.3^{C}	1156.7 ± 393.0^{BC}	0.007 ± 0.002^{B}	3.290 ± 0.664^{C}	1.787 ± 0.358^{C}
D. bermudensis (harbor)	400.0 ± 59.73^{C}	948.4 ± 147.3^{C}	0.008 ± 0.002^{B}	3.400 ± 1.139^{C}	1.608 ± 0.307^{C}
E. capsulatum (reef)	660.0 ± 133.9^{BC}	1347.4 ± 175.9^{BC}	0.011 ± 0.005^{AB}	7.229 ± 3.717^{BC}	2.649 ± 0.476^{B}
E. capsulatum (harbor)	815.0 ± 71.45^{B}	1536.0 ± 263.5^{B}	0.008 ± 0.006^{AB}	7.026 ± 6.129^{BC}	2.756 ± 0.615^{B}
Seawater (reef)	1319.7 ± 189.3^{A}	2845.9 ± 330.1^{A}	0.017 ± 0.002^{A}	22.66 ± 5.417^{A}	4.170 ± 0.221^{A}
Seawater (harbor)	1209.3 ± 8.505^{A}	2746.2 ± 113.5^{A}	0.010 ± 0.001^{AB}	11.60 ± 1.314^{B}	3.557 ± 0.085^{AB}

larval tunics, all of which indicate parent-to-offspring (vertical) symbiont transmission (Hirose, Oka and Akahori 2005; Hirose 2015; López-Legentil *et al.* 2015b). For the nonnative *D. bermudensis*, the stability of prokaryotic symbiont communities across different environments suggests that vertical transmission of symbionts may be the main acquisition mode for the species.

In contrast, for the native *E. capsulatum*, both host-specific and site-specific prokaryotic symbiont communities were detected, suggesting that horizontal transmission also occurred. Our study also revealed significant differences in the structure of the bacterioplankton communities between the two sites, potentially contributing to the differences observed in the symbiotic communities of *E. capsulatum*. Symbiont structure dynamics may reflect fluctuations in symbiont physiology and host-symbiont interactions across environmental conditions, with the microbiome of the nonnative host *D. bermudensis* exhibiting greater resilience, and the native host *E. capsulatum* greater susceptibility. However, given the dominance of both species across the different environment types examined, whether one approach is evolutionarily more advantageous than the other remains to be determined. Additional research is needed, including transplant field experiments and controlled aquaria experiments, in order to clarify the roles of symbiont acquisition and environmental tolerance on ascidian microbiome stability and host fitness.

Interestingly, the major OTU driving the observed dissimilarity between the symbiont communities in *E. capsulatum* specimens across sites was classified within the genus *Chelativorans*. *Chelativorans* spp. were also found but in relative low abundances in the seawater from both sites and in *D. bermudensis* samples. Species within this bacterial genus are known to be capable of degrading ethylenediaminetetraacetic acid (EDTA, Doronina *et al.* 2010). EDTA is a synthetic chemical compound widely utilized in detergents, water softeners and cosmetic products (Oviedo and Rodríguez 2003), and its prevalence in consumer products, along with its poor biodegradability and the inability of water treatment practices to remove EDTA from wastewater (Oviedo and Rodríguez 2003), results in high EDTA concentrations in coastal environments (Bedsworth and Sedlak 1999). As a chemical ligand, EDTA preferentially binds with heavy metals, resulting in the increased mobility and bioavailability of toxic heavy metals (Lo, Yang and Lin 1992). In addition, EDTA itself is considered an organic pollutant, and chronic exposure to EDTA has been shown to reduce the presence of critical mineral elements in the tissues of aquatic organisms (Nicula *et al.* 2011), while exposure to high concentrations of EDTA may result in mortality or inhibition of development in marine invertebrate larvae (Castille and Lawrence 1981). Thus, through an acquired capacity for processing pollutants like EDTA, symbiotic associations with microorganisms such as *Chelativorans* spp. may help explain how some ascidian species are able to flourish within polluted harbor environments.

Other bacterial taxa that contributed significantly to the observed dissimilarity between sites and species included the families *Rhodobacteraceae* and *Endozoicimonaceae*. *Rhodobacteraceae* are commonly described symbionts of marine invertebrates (Althoff *et al.* 1998), including ascidians (Erwin *et al.* 2014; Cahill *et al.* 2016; Evans *et al.* 2017), with links to nitrogen fixation and nitrate reduction (Pujalte *et al.* 2014). Similarly, members of the family *Endozoicimonaceae* are well-known ascidian symbionts (Cahill *et al.* 2016; Schreiber *et al.* 2016; Evans *et al.* 2017), with species capable of nitrate reduction (Kurahashi and Yokota 2007; Schreiber *et al.* 2016). If these capabilities result in enhanced host function, the presence of particular prokaryotic symbionts could be directly implicated in the successful establishment and spread of nonnative ascidians. However, as the presence of a particular symbiont capability does not necessarily translate to expressed function, the extent to which these putative symbiont functions extend to the holobiont remains unknown. Thus, further research utilizing metagenomic and metatranscriptomic datasets are clearly needed to fully characterize genes present in these communities, their functionality and their potential implications for host fitness and long-term survival in a new environment.

In summary, we describe two symbiont-mediated mechanisms that may allow ascidians to thrive in different environments: (1) Symbiont stability in some species (e.g. the nonnative *D. bermudensis*), where stable symbiotic communities maintained by vertical transmission are critical for host establishment and persistence across a broad range of abiotic factors, and (2) symbiont shuffling in other ascidian species (e.g. the native *E. capsulatum*), where the acquisition or a change in the relative abundance of specific prokaryotic symbionts (e.g. *Chelativorans*) may allow host ascidians to survive under particular local environmental conditions (e.g. EDTA pollution). Our results represent a significant advancement in our understanding of ascidian microbiomes and a critical step in assessing microbial contributions to ascidian ecology.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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REFERENCES

Althoff K, Schütt C, Steffen R *et al.* Evidence for a symbiosis between bacteria of the genus *Rhodobacter* and the marine sponge *Halichondria panicea*: Harbor also for putatively toxic bacteria ? *Mar Biol* 1998;**130**:529–36.

Altschul SF, Gish W, Miller W *et al.* Basic local alignment search tool. *J Mol Biol* 1990;**215**:403–10.

Bansal R, Mian MAR, Michel AP. Microbiome diversity of *Aphis glycines* with extensive superinfection in native and invasive populations. *Environ Microbiol Rep* 2014;**6**:57–69.

Bedsworth WW, Sedlak DL. Sources and environmental fate of strongly complexed nickel in estuarine waters: The role of ethylenediaminetetraacetate. *Environ Sci Technol* 1999;**33**:926–31.

Benjamini Y, Yekutieli D. The control of the false discovery rate in multiple testing under depencency. *Ann Stat* 2001;**29**:1165–88.

Bishop JDD, Sommerfeldt AD. Autoradiographic investigation of uptake and storage of exogenous sperm by the ovary

of the compound ascidian *Diplosoma listerianum*. *Mar Biol* 1996;**125**:663–70.
Bullard SG, Lambert G, Carman MR *et al*. The colonial ascidian *Didemnum* sp. A: Current distribution, basic biology and potential threat to marine communities of the northeast and west coasts of North America. *J Exp Mar Bio Ecol* 2007;**342**:99–108.
Burgsdorf I, Erwin PM, López-Legentil S *et al*. Biogeography rather than association with cyanobacteria structures symbiotic microbial communities in the marine sponge *Petrosia ficiformis*. *Front Microbiol* 2014;**5**:1–11.
Cahill PL, Fidler AE, Hopkins GA *et al*. Geographically conserved microbiomes of four temperate water tunicates. *Environ Microbiol Rep* 2016;**8**:470–8.
Caporaso JG, Lauber CL, Walters WA *et al*. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A* 2011;**108**:4516–22.
Carlton JT, Geller JB. Ecological roulette: The global transport of nonindigenous marine organisms. *Science* 1993;**261**:78–82.
Castille FL, Lawrence AL. The effects of EDTA (ethylenedinitrotetraacetic acid) on the survival and development of shrimp nauplii (*Penaeus stylirostris* Stimpson) and the interactions of EDTA with the toxicities of cadmium, calcium, and phenol. *J World Maric Soc* 1981;**12**:292–304.
Clarke KR, Gorley RN. *PRIMER v7: User Manual/Tutorial*. PRIMER-E, Plymouth 2015; 296pp.
Coats VC. Microbial associates of invasive *Berberis thunbergii* DC. *(Japanese barberry) Theses and Dissertations* 2013;**2050**:1–421.
Cohen AN, Carlton JT. Accelerating invasion rate in a highly invaded estuary. *Science* 1998;**279**:555–7.
de Bary A. Die Erscheinung der Symbiose: Vortrag gehalten auf der Versammlung deutscher Naturforscher und Aerzte zu Cassel. Strassburg. *Verlang von Karl J Trubner* 1879.
Devin S, Beisel JN. Biological and ecological characteristics of invasive species: A gammarid study. *Biol Invasions* 2006;**9**:13–24.
Doherty TS, Glen AS, Nimmo DG *et al*. Invasive predators and global biodiversity loss. *Proc Natl Acad Sci* 2016;**113**:11261–5.
Doronina NV, Kaparullina EN, Trotsenko YA *et al*. *Chelativorans multitrophicus* gen. nov., sp. nov. and *Chelativorans oligotrophicus* sp. nov., aerobic EDTA-degrading bacteria. *Int J Syst Evol Microbiol* 2010;**60**:1044–51.
Edgar RC, Haas BJ, Clemente JC *et al*. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 2011;**27**:2194–200.
Eichmiller JJ, Hamilton MJ, Staley C *et al*. Environment shapes the fecal microbiome of invasive carp species. *Microbiome* 2016;**4**:1–13.
Erwin PM, Carmen Pineda M, Webster N *et al*. Small core communities and high variability in bacteria associated with the introduced ascidian *Styela plicata*. *Symbiosis* 2013;**59**:35–46.
Erwin PM, Pineda MC, Webster N *et al*. Down under the tunic: bacterial biodiversity hotspots and widespread ammonia-oxidizing archaea in coral reef ascidians. *ISME J* 2014;**8**:575–88.
Evans JS, Erwin PM, Shenkar N *et al*. Introduced ascidians harbor highly diverse and host-specific symbiotic microbial assemblages. *Sci Rep* 2017;**7**:11033.
Folmer O, Black M, Hoeh W *et al*. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* 1994;**3**:294–9.
Fuhrman JA. Microbial community structure and its functional implications. *Nature* 2009;**459**:193–9.
Gantt SE, López-Legentil S, Erwin PM. Stable microbial communities in the sponge *Crambe crambe* from inside and outside a polluted Mediterranean harbor. *FEMS Microbiol Lett* 2017;**364**:1–7.
Gewing MT, Shenkar N. Monitoring the magnitude of marine vessel infestation by non-indigenous ascidians in the Mediterranean. *Mar Pollut Bull* 2017;**121:** 52–59.
Goodbody I. The ascidian fauna of a Jamaican lagoon: Thirty years of change. *Rev Biol Trop* 1993;**41**:35–8.
Grave B. Rate of growth, age at sexual maturity and duration of life of certain sessile organisms at Woods Hole, Massachusetts. *Biol Bull* 1933;**65**:375–86.
Hentschel U, Hopke J, Horn M *et al*. Molecular evidence for a uniform microbial community in sponges from different oceans. *Appl Environ Microbiol* 2002;**68**:4431–40.
Hirose E, Maruyama T. What are the benefits in the ascidian-*Prochloron* symbiosis ? *Endocytobiosis Cell Res* 2004;**15**:51–62.
Hirose E, Oka AT, Akahori M. Sexual reproduction of the photosymbiotic ascidian *Diplosoma virens* in the Ryukyu Archipelago, Japan: Vertical transmission, seasonal change and possible impact of parasitic copepods. *Mar Biol* 2005;**146**:677–82.
Hirose E. Ascidian photosymbiosis: Diversity of cyanobacterial transmission during embryogenesis. *Genesis* 2015;**53**:121–31.
Inderjit Chapman D, Ranelletti M, Kaushik S. Invasive marine algae: An ecological perspective. *Bot Rev* 2006;**72**:153–78.
Jackson JBC, Buss L. Allelopathy and spatial competition among coral reef invertebrates. *Source Proc Natl Acad Sci United States Am* 1975;**72**:5160–3.
Kearse M, Moir R, Wilson A *et al*. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 2012;**28**:1647–9.
Kozich JJ, Westcott SL, Baxter NT *et al*. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 2013;**79**:5112–20.
Kurahashi M, Yokota A. *Endozoicomonas elysicola* gen. nov., sp. nov., a γ-proteobacterium isolated from the sea slug *Elysia ornata*. *Syst Appl Microbiol* 2007;**30**:202–6.
Lambert CC, Lambert G. Non-indigenous ascidians in southern California harbors and marinas. *Mar Biol* 1998;**130**:675–88.
Lambert CC, Lambert G. Persistence and differential distribution of nonindigenous ascidians in harbors of the Southern California Bight. *Mar Ecol Prog Ser* 2003;**259**:145–61.
Lambert G. Ecology and natural history of the protochordates. *Can J Zool* 2005;**83**:34–50.
Lambert G. Invasive sea squirts: A growing global problem. *J Exp Mar Bio Ecol* 2007;**342**:3–4.
Lambert G. Marine biodiversity of Guam: the Ascidiacea. *Micronesica* 2003;**3536**:588–97.
Lambert G. Nonindigenous ascidians in tropical waters. *Pacific Sci* 2002;**56**:291–8.
Lee AH, Hooper-Bui L. The origin and *in situ* identification of uncultured gut bacteria in fourth-instar larvae of the red imported fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae). *Sociobiology* 2012;**59**:27–48.
Littman RA, Willis BL, Pfeffer C *et al*. Diversities of coral-associated bacteria differ with location, but not species, for three acroporid corals on the Great Barrier Reef. *FEMS Microbiol Ecol* 2009;**68**:152–63.
Lo KSL, Yang WF, Lin YC. Effects of organic matter on the specific adsorption of heavy metals by soil. *Toxicol Environ Chem* 1992;**34**:139–53.

López-Legentil S, Legentil ML, Erwin PM *et al.* Harbor networks as introduction gateways: contrasting distribution patterns of native and introduced ascidians. *Biol Invasions* 2015a;**17**:1623–38.

López-Legentil S, Turon X, Espluga R *et al.* Temporal stability of bacterial symbionts in a temperate ascidian. *Front Microbiol* 2015b;**6**:1–11.

Marins FO, Novaes RLM, Rocha RM *et al.* Non indigenous ascidians in port and natural environments in a tropical Brazilian bay. *Zool* 2010;**27**:213–21.

Martínez-García M, Stief P, Díaz-Valdés M *et al.* Ammonia-oxidizing *Crenarchaeota* and nitrification inside the tissue of a colonial ascidian. *Environ Microbiol* 2007;**10**:2991–3001.

Maruyama T, Hirose E, Ishikura M. Ultraviolet-light-absorbing tunic cells in didemnid ascidians hosting a symbiotic photooxygenic prokaryote, *Prochloron Biol Bull* 2003;**204**:109–13.

Mastrototaro F, Brunetti R. The non-indigenous ascidian *Distaplia bermudensis* in the Mediterranean: comparison with the native species *Distaplia magnilarva* and *Distaplia lucillae* sp. nov. *J Mar Biol Assoc UK* 2006;**86**:181–5.

McDonald D, Price MN, Goodrich J *et al.* An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* 2012;**6**:610–8.

McGee BL, Schlekat CE, Boward DM *et al.* Sediment contamination and biological effects in a Chesapeake Bay marina. *Ecotoxicology* 1995;**4**:39–59.

Megina C, González-Duarte MM, López-González PJ. Benthic assemblages, biodiversity and invasiveness in marinas and commercial harbours: an investigation using a bioindicator group. *Biofouling* 2016;**32**:465–75.

Mitchell CE, Power AG. Release of invasive plants from fungal and viral pathogens. *Nature* 2003;**421**:625–7.

Mumby PJ, Harborne AR, Brumbaugh DR. Grouper as a natural biocontrol of invasive lionfish. Gratwicke B (ed.). *PLoS One* 2011;**6**:e21510.

Nagar LR, Shenkar N. Temperature and salinity sensitivity of the invasive ascidian *Microcosmus exasperatus* Heller, 1878. *Aquat Invasions* 2016;**11**:33–43.

Nandakumar K, Tanaka M, Kikuchi T. Interspecific competition among fouling organisms in Tomioka Bay, Japan. *Mar Ecol Prog Ser* 1993;**94**:43–50.

Newton RJ, VandeWalle JL, Borchardt MA *et al.* *Lachnospiraceae* and *Bacteroidales* alternative fecal indicators reveal chronic human sewage contamination in an urban harbor. *Appl Environ Microbiol* 2011;**77**:6972–81.

Nicula M, Gergen I, Harmanescu M *et al.* Assessing the impact of EDTA chelating effect on some macro- and microminerals in Prussian carp (*Carassius gibelio*) tissues. *Sci Pap Anim Sci Biotechnol* 2011;**44**:40–4.

Nomaguchi T, Nishijima C, Minowa S *et al.* Embryonic thermosensitivity of the ascidian, *Ciona savignyi*. *Zoolog Sci* 1997;**14**:511–5.

Novak L, López-Legentil S, Sieradzki E *et al.* Rapid establishment of the non-indigenous ascidian *Styela plicata* and its associated bacteria in marinas and fishing harbors along the Mediterranean coast of Israel. *Med Mar Sci* 2017;**18**:324–31.

Olson RR. The consequences of short-distance larval dispersal in a sessile marine invertebrate. *Ecology* 1985;**66**:30–9.

Oviedo C, Rodríguez J. EDTA: The chelating agent under environmental scrutiny. *Quim Nova* 2003;**26**:901–5.

Parker MA, Malek W, Parker IM. Growth of an invasive legume is symbiont limited in newly occupied habitats. *Divers Distrib* 2006;**12**:563–71.

Peres JM. Ascidies récoltées dans les parages des Baléares par le 'Professeur Lacaze-Duthiers' (2e. partie: Iviza et San Antonio). *Vie Milieu* 1957;Suppl. 6: 223–34.

Pierce M. The Microbiome of the eastern oyster, *Crassostrea virginica* (Gmelin, 1791): Temporal and spatial variation, environmental influences, and its impact on host physiology. *Dr Diss* 2016;**1279**. https://opencommons.uconn.edu/dissertations/1279

Pineda MC, McQuaid CD, Turon X *et al.* Tough adults, frail babies: an analysis of stress sensitivity across early life-history stages of widely introduced marine invertebrates. *PLoS One* 2012;**7**:1–11.

Piola RF, Johnston EL. Pollution reduces native diversity and increases invader dominance in marine hard-substrate communities. *Divers Distrib* 2008;**14**:329–42.

Pita L, Turon X, López-Legentil S *et al.* Host rules: Spatial stability of bacterial communities associated with marine sponges (*Ircinia* spp.) in the western Mediterranean Sea. *FEMS Microbiol Ecol* 2013;**86**:268–76.

Pruesse E, Quast C, Knittel K *et al.* SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* 2007;**35**:7188–96.

Pujalte MJ, Lucena T, Ruvira MA *et al.* The family *Rhodobacteraceae*. *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria* 2014;**8**:439–512.

Renzi M, Perra G, Guerranti C *et al.* Assessment of environmental pollutants in ten southern Italy harbor sediments. *Toxicol Ind Health* 2009;**25**:351–63.

Richardson DM, Allsopp N, D'Antonio CM *et al.* Plant invasions - the role of mutualisms. *Biol Rev* 2000;**75**:65–93.

Rocha RM Da, Kremer LP. Introduced ascidians in Paranaguá Bay, Paraná, southern Brazil. *Rev Bras Zool* 2005;**22**:1170–84.

Rout ME, Chrzanowski TH, Westlie TK *et al.* Bacterial endophytes enhance competition by invasive plants. *Am J Bot* 2013;**100**:1726–37.

Ruiz GM, Carlton JT, Grosholz ED *et al.* Global invasions of marine and estuarine habitats by non-indigenous species: Mechanisms, extent, and consequences. *Integr Comp Biol* 1997;**37**:621–32.

Schiff K, Diehl D, Valkirs A. Copper emissions from antifouling paint on recreational vessels. *Mar Pollut Bull* 2004;**48**:371–7.

Schloss PD, Westcott SL, Ryabin T *et al.* Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009;**75**:7537–41.

Schloss PD, Westcott SL. Assessing and improving methods used in operational taxonomic unit-based approaches for 16S rRNA gene sequence analysis. *Appl Environ Microbiol* 2011;**77**:3219–26.

Schreiber L, Kjeldsen KU, Funch P *et al.* *Endozoicomonas* are specific, facultative symbionts of sea squirts. *Front Microbiol* 2016;**7**:1–15.

Shenkar N, Swalla BJ. Global diversity of Ascidiacea. Browman H (ed.). *PLoS One* 2011;**6**:e20657.

Simberloff D, Schmitz D, Brown T. *Strangers in Paradise: Impact and Management of Nonindigenous Species in Florida*. Island Press, 1997.

Simonsen AK, Dinnage R, Barrett LG *et al.* Symbiosis limits establishment of legumes outside their native range at a global scale. *Nat Commun* 2017;**8**:1–9.

Sims LL. Osmoregulatory capabilities of three macrosympatric stolidobranch ascidians, *Styela clava* Herdman, *S. plicata*

(Lesueur), and *S. montereyensis* (Dall). *J Exp Mar Bio Ecol* 1984;**82**:117–29.

Stabili L, Licciano M, Longo C *et al*. The Mediterranean non-indigenous ascidian *Polyandrocarpa zorritensis*: Microbiological accumulation capability and environmental implications. *Mar Pollut Bull* 2015;**101**:146–52.

Stefaniak L, Lambert G, Gittenberger A *et al*. Genetic conspecificity of the worldwide populations of *Didemnum vexillum* Kott, 2002. *Aquat Invasions* 2009;**4**:29–44.

Torchin ME, Lafferty KD, Dobson AP *et al*. Introduced species and their missing parasites. *Nature* 2003;**421**:628–30.

Turner SJ, Thrush SF, Cummings VJ *et al*. Changes in epifaunal assemblages in response to marina operations and boating activities. *Mar Environ Res* 1997;**43**:181–99.

Turon X, Nishikawa T, Rius M. Spread of *Microcosmus squamiger* (Ascidiacea: Pyuridae) in the Mediterranean Sea and adjacent waters. *J Exp Mar Bio Ecol* 2007;**342**:185–8.

van Name WG. The ascidians of the Bermuda Islands. *Trans Connect Acad Sci* 1902;**11**:325–412.

van Name WG. The North and South American ascidians. *Bull Am Museum Nat Hist* 1945;**84**:1–476.

Villalobos SM, Lambert G, Shenkar N *et al*. Distribution and population dynamics of key ascidians in North Carolina harbors and marinas. *Aquat Invasions* 2017;**12**:447–58.

Vrijenhoek RC. Genetics and evolution of deep-sea chemosynthetic bacteria and their invertebrate hosts. *The Vent and Seep Biota, Topics in Geobiology*. Vol **33**. Springer Netherlands, 2010, 15–49.

Wang Q, Garrity GM, Tiedje JM *et al*. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007;**73**:5261–7.

Warton DI, Wright ST, Wang Y. Distance-based multivariate analyses confound location and dispersion effects. *Methods Ecol Evol* 2012;**3**:89–101.

White JR, Nagarajan N, Pop M. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. *PLoS Comput Biol* 2009;**5**:e1000352.

Zhan A, Briski E, Bock DG *et al*. Ascidians as models for studying invasion success. *Mar Biol* 2015;**162**:2449–70.

Zhang R, Lau SCK, Ki JS *et al*. Response of bacterioplankton community structures to hydrological conditions and anthropogenic pollution in contrasting subtropical environments. *FEMS Microbiol Ecol* 2009;**69**:449–60.