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Research Letter

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Stable microbial communities in the sponge *Crambe crambe* from inside and outside a polluted Mediterranean harbor

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One sentence summary: The microbiome of the common Mediterranean sponge, *Crambe crambe*, exhibited greater stability and pollution tolerance than free-living microbial counterparts.

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ABSTRACT

Marine sponges have been shown to harbor diverse microbial symbiont communities that play key roles in host functioning, yet little is known about how anthropogenic disturbances impact sponge–microbe interactions. The Mediterranean sponge *Crambe crambe* is known to accumulate heavy metals in polluted harbors. In this study, we investigated whether the microbiome of *C. crambe* differed between sponges inhabiting a polluted harbor in Blanes (Spain) and a nearby (<1 km) natural environment. Triplicate sponge and ambient seawater samples were collected from each site and the microbial composition of each sample was determined by 16S rRNA gene sequence analysis (Illumina Hi-Seq platform). No significant differences in the diversity or structure of microbial communities in *C. crambe* were detected between habitats, while a significant difference in community structure was observed in ambient seawater inside and outside of the polluted harbor. The microbiome of *C. crambe* was clearly differentiated from free-living seawater microbes and dominated by *Proteobacteria*, specifically a single betaproteobacterium that accounted for >86% of all sequence reads. These results indicate that sponge microbiomes exhibit greater stability and pollution tolerance than their free-living microbial counterparts, potentially mitigating the effects of pollutants on coastal marine communities.

Keywords: microbiome; heavy metals; low microbial abundance (LMA); intraspecific variation; bioindicator; anthropogenic effects

INTRODUCTION

Sponges (phylum Porifera) are an ancient metazoan taxon, appearing in the fossil record as early as 580 million years ago (Li, Chen and Hua 1998). Throughout most of their evolutionary history, sponges have lived in association with diverse microorganisms and today host abundant and complex microbial communities (Taylor *et al.* 2007, 2013; Thomas *et al.* 2010, 2016; Hentschel *et al.* 2012). In fact, some sponge species are so laden with microbes that bacterial and archaeal symbionts account for as much as 38% of sponge biomass (Vacelet 1975).

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Sponge-associated microbial communities differ from the free-living communities of seawater and sediments (Hentschel *et al.* 2002; Weigel and Erwin 2016) and are more similar within a species, even across broad geographical ranges, than between sponge species (Hentschel *et al.* 2002; Lee *et al.* 2011). To date, however, there have been few studies that have investigated the drivers of intraspecific variation in sponge microbiomes, such as host health or differences in key environmental factors. For example, diseased sponges exhibited alterations in their microbial community structure compared to healthy sponges, possibly resulting from direct effects of the disease-causing microbes and indirect effects of nutrient release from degraded sponge tissues (Webster *et al.* 2002, 2008). Temperature extremes have also been shown to alter sponge-associated microbial communities (Lemoine *et al.* 2007; Webster, Cobb and Negri 2008), while natural variations across seasons have little impact on sponge microbiome structure (Erwin *et al.* 2012, 2015). Similarly, changes in sponge-associated microbial communities have been reported in response to some aspects of environmental pollution (e.g. heavy metals), but not others (e.g. nitrogen pulses, Luter, Gibb and Webster 2014), with Webster *et al.* (2001) showing that copper exposure caused changes in the microbial community of the Indo-Pacific sponge, *Rhopaloeides odorabile*. A similar effect of copper exposure on sponge microbiome composition was subsequently reported by Tian *et al.* (2014), suggesting that heavy metals significantly impact sponge-associated microbial communities in disparate hosts.

Sponges are efficient filter-feeders (Reiswig 1971; Turon, Galera and Uriz 1997; Ribes, Coma and Gili 1999; McMurray, Pawlik and Finelli 2014) and have the ability to overturn the entirety of the overhead water column on shallow reefs within 24 h (Reiswig 1974) and to retain up to 80% of the particulate matter they filter (Milanese *et al.* 2003). Sponges also consume dissolved organic matter (e.g. McMurray *et al.* 2016), including dissolved organic carbon released by neighboring reef inhabitants (Rix *et al.* 2016), and retain this diffuse nutrient pool in reef systems as biomass or particulate organic matter (de Goeij *et al.* 2013). Due to their ability to pump large amounts of ambient seawater through their high-volume filter-feeding system and their inability to escape waterborne pollution (Carballo and Naranjo 2002; Rosenberg *et al.* 2004; Perez *et al.* 2005), sponges represent ideal candidates for use in bioremediation (Milanese *et al.* 2003) and as biomonitors (Cebrian, Uriz and Turon 2007). The use of sponges as biomonitors for heavy metal pollution was investigated in the NW Mediterranean Sea (Catalonia, Spain) and the common sponge species *Crambe crambe* was identified as the most accurate bioindicator for even small fluctuations in heavy metal pollution (Cebrian, Uriz and Turon 2007). In fact, high amounts of heavy metals (copper and lead) accumulated within the tissues of *C. crambe* collected from inside harbors, while sponges collected from adjacent, natural habitats exhibited low amounts of heavy metals (i.e. levels similar to sponges collected from pristine areas in the region, Cebrian, Uriz and Turon 2007). As such, this system offers a unique opportunity to investigate the effects of polluted habitats on sponge-associated microbes and provides further insight into the effectiveness of *C. crambe* as a bioindicator species. Here, we assessed the effects of anthropogenically disturbed habitats on the diversity, composition and structure of microbial symbionts associated with the orange–red encrusting sponge *C. crambe*. To achieve this goal, we characterized and compared microbial communities in *C. crambe* and seawater collected from a polluted harbor (Blanes Harbor) and an adjacent (<1 km), natural habitat outside the harbor (Punta Santa Anna).

METHODS

Sample collection

Triplicate samples of *Crambe crambe* (Schmidt 1862) and ambient seawater (1 L) were collected at two locations separated by <1 km: inside Blanes Harbor (BH, 41.6742°N, 2.7992°E) and at Punta Santa Anna (SA, 41.6725°N, 2.8038°E) in October 2012. Sediment and sponge samples previously collected from BH exhibited high copper (97.7 and 153.2 μg/g) and lead (69.0 and 4.2 μg/g, respectively) levels compared to SA (copper: 6.0 and 13.2 μg/g, lead: 31.0 and 2.4 μg/g, respectively; identified as 'Blanes' in Cebrian, Uriz and Turon 2007). Sponge and seawater samples were transported in an insulated cooler to the laboratory (3.5 km away) and processed within 1 h of sampling. All sponges were collected in separate sterile bags, rinsed with filtered seawater and preserved in 100% ethanol before being stored at –20°C. Seawater samples were immediately concentrated on 0.2 μm filters and then stored at –80°C.

DNA extraction and sequence processing

DNA extracts were prepared from sponge ($n = 6$) and seawater ($n = 6$) samples using the Powersoil DNA Extraction kit (MoBio, Carlsbad, CA, USA) following the Earth Microbiome Project standard protocols (http://press.igsb.anl.gov/earthmicrobiome/emp-standard-protocols/dna-extraction-protocol/). Partial (V4) 16S rRNA gene sequences were amplified using the forward primer 515f and reverse primer 806r and sequenced on a HiSeq2500 platform (Illumina, San Diego, CA, USA) at the University of Colorado, Boulder, CO, USA, following the Earth Microbiome Project standard procedures (http://www.earthmicrobiome.org/emp-standard-protocols/16s/, Caporaso *et al.* 2011). Illumina sequence reads were processed in mothur (Schloss *et al.* 2009) as described previously (Thomas *et al.* 2016). Briefly, raw sequences were demultiplexed, quality-filtered, aligned, classified and clustered into operational taxonomic units (OTUs) at 97% sequence identity. To equalize sequencing depth among samples (range = 14 181–25 698 reads), each dataset was subsampled to the lowest read count ($n = 14\,181$). Initially identified as an unclassified proteobacterium, the dominant OTU (1585) was further classified to the class *Betaproteobacteria* following an identical match (100%) to a previously reported symbiont from *C. crambe* at the same rank (GenBank Acc. No. KC492702, Croué *et al.* 2013). Sequence and metadata are available under study 1740 at the following portal: http://qiita.microbio.me/.

Data analysis

Microbial community diversity

To compare the microbial diversity of seawater and *C. crambe* for the two sampling sites, mothur (version 1.36.1, Schloss *et al.* 2009) was used to calculate the OTU richness (S), Shannon-Weaver diversity index (H′) and Simpson index (D). Two-way crossed analyses of variance (ANOVA) were run to test for significant differences in diversity indices across the factors source (sponge vs. seawater), location (inside vs. outside harbor) and an interaction term. Tukey's honest significant difference (HSD) tests were run for multiple post-hoc comparisons of means.

Microbial community structure

Bray–Curtis similarity matrices were constructed using OTU relative abundance data in PRIMER (version 6.1.11) and visualized in a cluster dendrogram. Permutational multivariate analyses

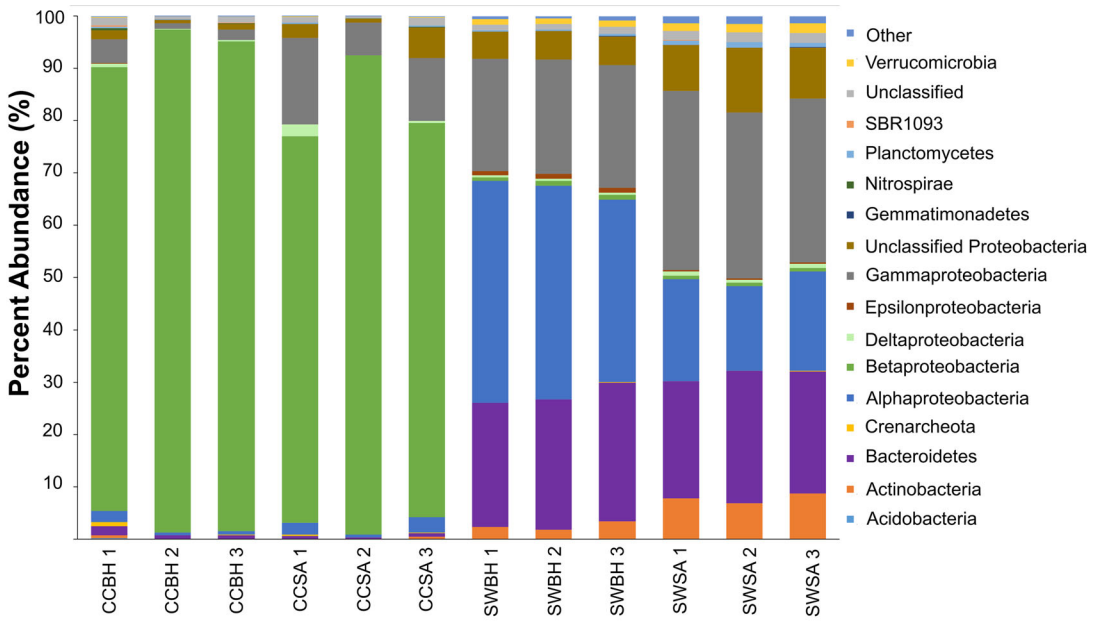

Figure 1. Phylum level composition of microbial communities in the sponge *Crambe crambe* (CC) and ambient seawater (SW) from a natural habitat (Punta Santa Anna, SA) and heavy metal-contaminated harbor (Blanes Harbor, BH). Sequences affiliated with *Proteobacteria* are further divided into the class level.

of variance (PERMANOVA, version 1.0.1) were performed to test for differences in community structure across source (sponge vs. seawater), location (inside vs. outside harbor) and an interaction term. Pairwise comparisons were conducted among levels within the interaction term, with significance determined by Monte Carlo asymptotic *P*-values.

OTU level analyses

To determine the contribution of individual OTUs to the overall dissimilarity between communities, a similarity percentages (SIMPER) analysis was conducted using OTU relative abundance matrices in PRIMER, with a cutoff percentage of 0.70. To determine significant differences in OTU relative abundances between locations for dominant *C. crambe* microbial taxa, Metastats (White, Nagarajan and Pop 2009) was performed in mothur with 1000 permutations. Basic Local Alignment Search Tool (BLAST) analyses were conducted with 16S rRNA nucleotide sequences of the 10 most common OTUs in *C. crambe* to compare symbiont sequences herein to those in the GenBank database, recording percentage identity and sources of top matches.

RESULTS

Comparative analysis of microbial community diversity and composition

Of the total 6434 OTUs recovered, 2271 were associated with the sponge *Crambe crambe* and 4170 were detected in the seawater samples. The OTUs recovered represented 33 bacteria and 3 archaea phyla. Of the three archaea phyla observed, *Crenarchaeota* was found in all samples, while *Euryarchaeota* and *Parvarchaeota* were only observed in the seawater samples (Fig. 1). Of the bacteria phyla, 28 and 29 phyla were observed in *C. crambe* and seawater, respectively (Fig. 1). Microbial communities in *C. crambe* and seawater were dominated by the phylum *Proteobacteria* (98% and 66%, respectively, Fig. 1). However, in *C. crambe*, *Betaproteobacteria* was the dominant class while seawater samples were dominated by *Alphaproteobacteria* and *Gammaproteobacteria* (Fig. 1).

Table 1. Alpha diversity measurements of microbial communities in the sponge *C. crambe* and seawater samples from Blanes Harbor (BH) and Punta Santa Anna (SA). Values are means ± 1 standard error. Different letters indicate significant differences based on Tukey's HSD tests. S = OTU richness, H′ = Shannon-Weaver Diversity and D = Simpson Diversity Index.

Source	S	H′	D
SA *C. crambe*	473 ± 103^{A}	1.30 ± 0.351^{A}	0.653 ± 0.0941^{A}
BH *C. crambe*	663 ± 209^{A}	0.757 ± 0.300^{A}	0.839 ± 0.0626^{A}
SA Seawater	2383 ± 179^{B}	5.87 ± 0.0378^{B}	0.0103 ± 0.000249^{B}
BH Seawater	1873 ± 134^{B}	5.22 ± 0.109^{B}	0.0265 ± 0.00274^{B}

All diversity indices (H′, D and S) were significantly different (ANOVA, $P < 0.0001$) between the sampled sources (seawater and *C. crambe*), with higher diversity in seawater microbial communities compared to *C. crambe* (Table 1). No significant effects of location (except H′, ANOVA, $P = 0.0370$) or interaction terms (location*source) were detected. Accordingly, pairwise post-hoc tests revealed no significant differences in diversity metrics across locations within each source (seawater and *C. crambe*, Table 1).

Comparative analysis of microbial community structure

Microbial communities in seawater and *C. crambe* clustered by source, with seawater communities further clustering by location within source (Fig. 2). Accordingly, microbial community structure was significantly different between seawater and *C. crambe* (PERMANOVA, $P = 0.001$), with the factor source accounting for most (82.9%) of the variance among samples. The factor location (inside vs. outside harbor) and the interaction term (location*source) were also significant (PERMANOVA, $P = 0.002$ and 0.001, respectively), driven by significant differences in seawater microbial community structure between locations

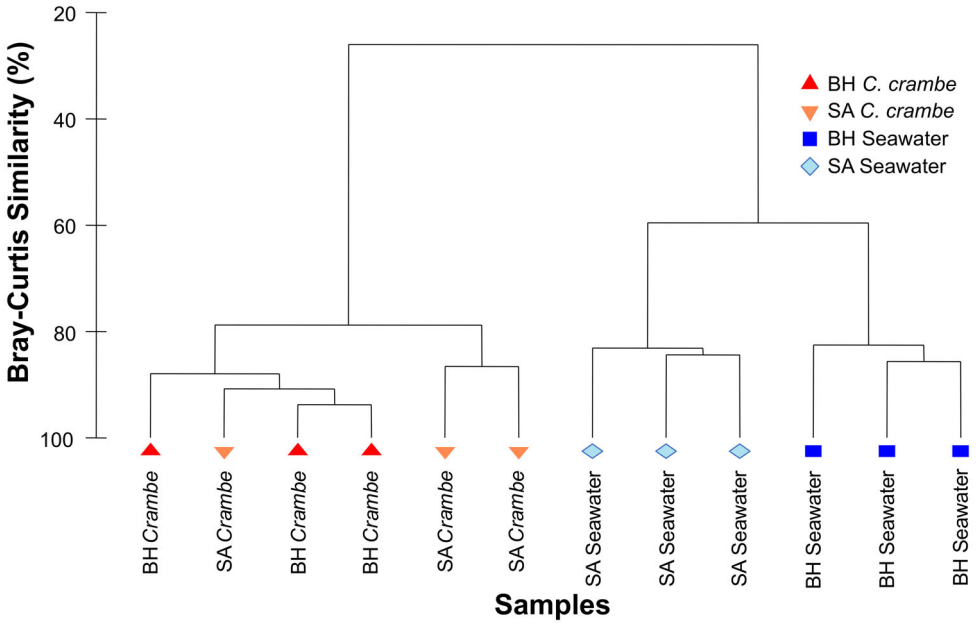

Figure 2. Cluster dendrogram based on Bray–Curtis dissimilarity matrices of microbial communities in the sponge *Crambe crambe* (CC) from Blanes Harbor (BH, red triangles) and Punta Santa Anna (SA, orange triangles), and ambient seawater (SW) from Blanes Harbor (BH, dark blue squares) and Punta Santa Anna (SA, light blue diamonds).

(PERMANOVA, $P = 0.004$). In contrast, microbial communities in *C. crambe* did not differ in overall structure across locations (PERMANOVA, $P = 0.115$).

In total, 10 OTUs contributed to 70% of the 17.66% observed overall community dissimilarity between the microbiomes of *C. crambe* collected inside and outside the harbor (Table 2). These OTUs were all classified to the phylum *Proteobacteria* and one OTU (OTU1585) accounted for the majority (52.46%) of the dissimilarity in microbiome structure between locations (Table 2). This betaproteobacterial OTU comprised 91.36% and 80.94% of sequence reads from *C. crambe* inside and outside the harbor, respectively, and was detected in low abundance (<0.1%) within seawater microbial communities. Of the remaining nine OTUs, five were found exclusively in *C. crambe* samples and three were found in low abundance (<0.1%) within one or two replicates of seawater samples (Fig. 2; Table 2). Notably, eight of these OTUs were more abundant in the microbiome of *C. crambe* from outside the harbor compared to *C. crambe* samples collected in the harbor, with four OTUs exhibiting significantly different relative abundances (Metastats, $P < 0.05$, Table 2). BLASTn results showed that 5 of the top 10 OTUs were most closely matched (97%–100% identity) to *Proteobacteria* associated with other specimens of *C. crambe* and other sponge species (Table 2), while the remaining five OTUs matched closely (98%–100%) to *Proteobacteria* associated with corals, seawater, or sediment samples (Table 2).

DISCUSSION

This study demonstrated that the microbial community of *Crambe crambe* distinctly differs in composition and structure from the free-living community observed in ambient seawater, consistent with previous studies of sponge microbiomes (Lee *et al.* 2011; Hentschel *et al.* 2012; Jackson *et al.* 2012). Further, our analyses revealed greater stability of sponge-associated microbial communities across habitats impacted by heavy metal pollution compared to seawater communities. No significant differences in microbial symbiont diversity and structure were observed between *C. crambe* sponges collected inside and outside of a polluted harbor, indicating no strong effect of heavy metal pollution on the *C. crambe* microbiome. In contrast, seawater communities exhibited a significant difference in structure across these same habitats, indicating a shift in bacterioplankton composition in response to different environmental conditions within the harbor. Similar shifts in free-living and some host-associated microbial communities have been reported in anthropogenically impacted habitats, including heavy metal contamination. For example, shallow coastal corals in polluted habitats exhibited significant differences in microbial community composition compared to corals living in less polluted areas (Klaus *et al.* 2007), and small shifts in the abundance of *Acidobacteria* and *Gammaproteobacteria* were associated with heavy metal-polluted marine sediments (Zhao *et al.* 2014). In sponges, field studies have reported correlations between heavy metal concentrations (zinc, nickel, lead and copper) in the environment and the structure of sponge-associated microbial communities (e.g. Bauvais *et al.* 2015), as well as, direct impacts of copper concentrations on symbiont structure and function in experimental settings (Webster *et al.* 2001; Tian *et al.* 2014). As such, the stability of the microbiome in *C. crambe* across natural and heavy metal-impacted locations is particularly notable and suggests that not all sponge-associated microbial communities are disrupted by exposure to pollution and heavy metals.

Crambe crambe has been categorized as a low microbial abundance (LMA) sponge via electron microscopy (Gloeckner *et al.* 2014) and, similar to other LMA sponges, the microbial community associated with *C. crambe* samples herein was dominated by *Proteobacteria* (Webster and Hill 2001; Croué *et al.* 2013; Giles *et al.* 2013; Gloeckner *et al.* 2014). In particular, a single betaproteobacterial OTU comprised on average 86.15% of the microbial community in *C. crambe* and matched identically to the dominant betaproteobacterium reported previously in *C. crambe* from the Mediterranean coast of France (Croué *et al.* 2013), located

Table 2. Taxonomic classification and top BLASTn matches (sources in parenthesis, sponge species names in italics) of common bacterial OTUsin the sponge *C. crambe*. OTUs are ranked by percentage contributions to dissimilarity (SIMPER analysis) between collection locations and OTU relative abundances in *C. crambe* are shown by location, Blanes Harbor (BH) and Punta Santa Anna (SA). Asterisks (*) indicate significantly different (Metastats, $P < 0.05$) OTU abundances between locations.

OTU	Phylum	Top BLASTn match (source)	Accession number	Percent identity	% Contribution to dissimilarity	Metastats P-value	Relative abundance BH	Relative abundance SA
1585	*Proteobacteria*	Uncultured betaproteobacterium (*Crambe crambe*)	KC492702	100	52.46	0.1770	91.36	80.94
2273	*Proteobacteria*	Uncultured alphaproteobacterium (*Axinella verrucosa*)	AJ581348	100	2.94	0.1647	0.08	1.41
124222	*Proteobacteria*	*Endozoicomonas montiporae* (Coral)	CP013251	100	2.62	0.0293*	0.00	1.25
254338	*Proteobacteria*	Uncultured gammaproteobacterium (*Ianthella basta*)	JN388027	100	2.56	0.0038*	0.00	1.25
1007	*Proteobacteria*	*Spongiobacter* sp.(*Rhabdastrella globostellata*)	KF282425	99	1.99	0.0043*	0.12	1.11
216271	*Proteobacteria*	Uncultured betaproteobacterium (*Crambe crambe*)	KC492701	97	1.90	0.0177*	0.46	1.43
28461	*Proteobacteria*	Uncultured proteobacterium (Seawater)	JN974071	100	1.89	0.2609	0.06	0.97
251760	*Proteobacteria*	Uncultured gammaproteobacterium (Seawater)	LC087332	98	1.45	0.1655	0.03	0.71
2442	*Proteobacteria*	Uncultured bacterium(Coastal sediment)	JQ257827	99	1.29	0.2372	0.00	0.63
136112	*Proteobacteria*	Uncultured alphaproteobacterium (Seawater)	HM474883	98	1.08	0.9204	0.69	0.50

150 km from our sampling location in Blanes, Spain, that has eluded cultivation (Öztürk *et al.* 2013). The shared presence and dominance of this symbiont in geographically disparate hosts suggests that the main components of microbial communities in *C. crambe* are stable across geographic areas and varying environmental conditions, as reported for other Mediterranean sponges (e.g. Pita *et al.* 2013b). An additional nine common proteobacterial OTUs in *C. crambe* represented microbial taxa specific to *C. crambe*, present in other sponge species (e.g. *Axinella verrucosa*, *Ianthella basta* and *Rhabdastrella globostellata*), or detected in unrelated hosts (e.g. corals) and environmental samples (e.g. seawater, sediment). Notably, all nine of these common symbiont taxa were more abundant in the microbiomes of *C. crambe* from the natural habitat compared to *C. crambe* from the impacted harbor, possibly indicating minor and OTU-specific impacts of heavy metal pollution on the abundance of common symbiont taxa in *C. crambe*.

Overall, the results of our study indicate that the microbiome of *C. crambe* exhibits greater stability and pollution tolerance than free-living bacterioplankton and suggest that some sponges may be able to mitigate the effects of heavy metal pollutants through persistent relationships with microbial symbionts. Indeed, previous research has shown seasonal fluctuations of copper and lead levels in *C. crambe* tissues (Cebrian, Uriz and Turon 2007), indicating that this sponge may have some mechanism to process and eliminate heavy metals from its body. Further, bacteria resistant to heavy metal pollution have been isolated from sponges (Selvin *et al.* 2009) and enzymes capable of detoxifying heavy metals have been detected in sponge symbiont communities (e.g. mercuric reductase proteins, Fan *et al.* 2012). While additional research is required to document microbial-mediated processing of heavy metals in sponges, the observed maintenance of stable microbial communities in *C. crambe* collected inside and outside a polluted harbor is notable, as these conditions have been shown to affect other aspects of sponge biology. Individuals of *C. crambe* transplanted from natural habitat to Blanes Harbor exhibited reduced growth, fecundity and survival compared to controls, responses attributed to the effects of elevated heavy metal concentrations (Cebrian *et al.* 2003). It is possible that the concentrations of heavy metals or other pollutants in the investigated harbor were not high enough to cause a significant impact on the microbial community of *C. crambe*; however, previous reports of copper levels in *C. crambe* tissue from these same harbors (ca. 150 μg/g, Cebrian, Uriz and Turon 2007) were similar to levels that induced symbiont shifts in other sponge species (Webster *et al.* 2001) and the investigated harbor contained the highest levels of lead and second highest levels of copper among 16 harbors across ca. 250 km of Mediterranean coastline (Cebrian, Uriz and Turon 2007). Finally, our results indicate that the microbial community of *C. crambe* is not a useful bioindicator, due to the observed stability under moderate pollution stress, yet may have potential applications in bioremediation via heavy metal detoxification. Future studies targeting how heavy metal exposure affects functional aspects of microbial communities in *C. crambe* (e.g. gene presence and expression) are needed to elucidate potential mechanisms of heavy metal resistance and detoxification in sponge holobionts.

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Conflict of interest. None declared.

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