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Divergent morphological and microbiome strategies of two neighbor sponges to cope with low pH in Mediterranean CO₂ vents

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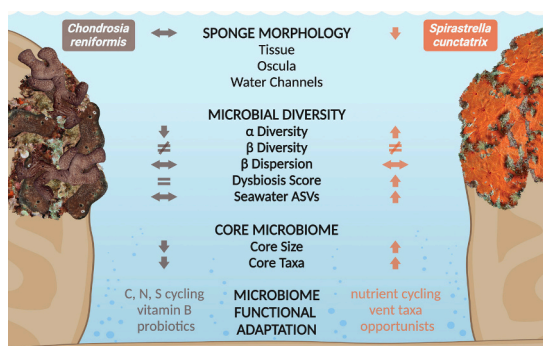
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HIGHLIGHTS

- Sponges are seen as winner taxa of future OA, yet not all species respond equally.
- Neighbor sponges had different morphology and microbiome patterns in CO₂ vents.
- Vent *S. cunctatrix* displayed morphology changes and incipient microbial dysbiosis.
- *C. reniformis* microbiomes were normobiotic, diverse and functionally flexible.
- Symbioses supplying C–N–S cycles, vitamins and probiotics uphold resilience to OA.

GRAPHICAL ABSTRACT



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ABSTRACT

Ocean Acidification (OA) profoundly impacts marine biochemistry, resulting in a net loss of biodiversity. Porifera are often forecasted as winner taxa, yet the strategies to cope with OA can vary and may generate diverse fitness status. In this study, microbial shifts based on the V₃–V₄ 16S rRNA gene marker were compared across neighboring *Chondrosia reniformis* sponges with high microbial abundance (HMA), and *Spirastrella cunctatrix* with low microbial abundance (LMA) microbiomes. Sponge holobionts co-occurred in a CO₂ vent system with low pH (pHT ~ 7.65), and a control site with Ambient pH (pHT ~ 8.05) off Ischia Island, representing natural analogues to study future OA, and species' responses in the face of global environmental change. Microbial diversity and composition varied in both species across sites, yet at different levels. Increased numbers of core taxa were detected in *S. cunctatrix*, and a more diverse and flexible core microbiome was reported in *C. reniformis* under OA. Vent *S. cunctatrix* showed morphological impairment, along with signs of putative stress-induced dysbiosis, manifested by: 1) increases in alpha diversity, 2) shifts from sponge related microbes towards seawater microbes, and 3) high dysbiosis scores. *Chondrosia reniformis* in lieu, showed no morphological variation, low dysbiosis scores, and experienced a reduction in alpha diversity and less number of core taxa in vent specimens. Therefore,

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C. reniformis is hypothesized to maintain a state of normobiosis and acclimatize to OA, thanks to a more diverse, and likely metabolically versatile microbiome. A consortium of differentially abundant microbes was identified associated to either vent or control sponges, and chiefly related to carbon, nitrogen and sulfur-metabolisms for nutrient cycling and vitamin production, as well as probiotic symbionts in *C. reniformis*. Diversified symbiont associates supporting functional convergence could be the key behind resilience towards OA, yet specific acclimatization traits should be further investigated.

1. Introduction

Sponges are predicted to be winners in future scenarios of global warming and ocean acidification (OA), as they seem more tolerant to a range of stressful conditions compared to other benthic organisms, and may benefit from a reduced spatial competition (Bell et al., 2018a, 2018b). Consequent benthic community phase shift predictions led to the formulation of the “vicious cycle” hypothesis, in which expanding sponge and macroalgal populations would reciprocally promote their prevalence (Pawlik et al., 2016). In colonizing challenging environments, sponges benefit from flexible nutritional strategies (Hadas et al., 2009; De Goeij et al., 2013), being able to take up different forms of organic matter, including dissolved organic matter (DOM) (De Goeij et al., 2013; De Goeij et al., 2017; Morganti et al., 2017). They further obtain energy and nutrients from chemo- and photoautotrophic symbionts (Cheshire and Wilkinson, 1991; Nakagawa and Takai, 2008; Ribes et al., 2012; Feng and Li, 2019). Nonetheless, recent perspectives based on models forecasting net reductions on primary productivity, argue that Porifera could be penalized by climate change (rather than favoured; Lesser and Slattery, 2020), due to limiting resources via negative bottom-up effects (Lesser and Slattery, 2013, 2020; De Goeij et al., 2017; Morganti et al., 2017). OA is defined as the change in carbonate chemistry resulting from the uptake of atmospheric CO₂ by the ocean, along with the decrease of seawater pH and the calcium carbonate saturation states (Orr et al., 2005). Calcifying organisms that build on calcareous skeletons or shells, including scleractinian corals, bryozoans, echinoderms, molluscs, calcareous algae, are remarkably impaired under acidified conditions, as calcification is expected to decrease as seawater acidity increases (Doney et al., 2009). Sponges with calcareous spicules (class Calcarea) include few species, being OA quite innocuous for the silicic skeletons of the majority of Porifera (classes Demospongiae, Hexactinellida, Homoscleromorpha) (Van Soest et al., 2012; Vicente et al., 2016). On what concerns the largest Porifera class –Demospongiae (Van Soest et al., 2012), bioeroding species will probably be favoured by low pH (Wisshak et al., 2014). Little adverse effect of OA alone, has been described in an array of sponges by in laboratory conditions (Bennett et al., 2017; Botté et al., 2019). But in combination with elevated temperatures heterotrophic species were significantly sensitive, whereas hosts harbouring phototrophic symbionts, reported mitigation from thermal stress in concomitance with elevated pCO₂ (Bennett et al., 2017). In other aquarium studies, elevated pCO₂ in combination with elevated temperatures, reported impacts on sponge microbiomes, with OA causing instability in microbial compositions (Lesser et al., 2016); and also tissue necrosis, dysbiosis and metabolic disfunctions (Botté et al., 2019). In situ surveys inform of negative effects (Fabricius et al., 2011), but also OA tolerance in diverse sponge assemblages (Carballo and Bell, 2017; Bates and Bell, 2018). All and all, not all species are equally resilient to OA, and it is unclear to what extent sponge-associated microbiomes are involved in possible mitigation processes.

The symbiotic relationships established with bacteria, archaea, fungi, protists, and viruses turn Porifera into model holobionts (Margulis, 1990; Rohwer et al., 2002; Bordenstein and Theis, 2015; Moitinho-Silva et al., 2017). These partnerships play crucial roles in promoting niche expansion, by introducing biomolecular scaffolds to host metabolism, and participating in nutrient cycling (Diaz and Rützler, 2001; Mohamed et al., 2008; Hudspeth et al., 2021; Lesser et al., 2022). Endosymbionts also produce secondary metabolites that deter predators (Pawlik et al., 1995; Schupp et al., 1999; Becerro et al., 2003; Sabrina

Pankey et al., 2022) or suppress the growth of competing organisms (Sullivan et al., 1983; Porter and Targett, 1988; Turon et al., 1996). They also provide essential vitamins (Fan et al., 2012; Hallam et al., 2006; Liu et al., 2012; Siegl et al., 2011; Thomas et al., 2010), and probiotic taxa can modulate inter-microbe interactions for compositional balancing (Cavallo et al., 2021). All these functions support overall performance and reproductive success of their hosts (Webster and Taylor, 2012; Freeman et al., 2014). Hence, evaluating sponge microbiomes' dynamics can unravel their contribution in holobionts' health, and in their responses to environmental changes (Costello et al., 2012; Fierer et al., 2012; Sommer et al., 2017; Pita et al., 2018).

Based on the abundance in symbiotic microorganisms, sponges can be classified into two categories named High microbial abundance (HMA) and Low microbial abundance (LMA). HMA species harbour very dense and diverse microbial consortia (Reiswig, 1974; Vacelet and Donadey, 1977; Wilkinson, 1978) containing between 10⁸ and 10¹⁰ microorganism cells per gram of sponge, and comprising 20–35 % of their total biomass (Reiswig, 1981; Webster et al., 2001; Hentschel et al., 2012). LMA sponges instead contain between 10⁵ and 10⁶ microbial cells per gram of sponge in lower taxonomic diversity (Hentschel et al., 2006). Microbes that are vertically inherited through parental gametes or brooded embryos (Buchner, 1965; Mira and Moran, 2002), significantly shape sponge microbiomes during reproduction. Additionally, symbionts can be acquired from the surrounding environment in post-metamorphosis stages, coinciding with the formation of the filtration system (Björk et al., 2019; Carrier et al., 2022; Turon et al., 2023). In general, sponges present mixed vertical and horizontal symbiont transmission modes at different ratios, according to host evolutionary history, health status or habitat conditions (Diez-Vives et al., 2022).

The HMA-LMA dichotomy is associated with morphological differences, with HMA hosts being better adapted to accumulate larger microorganismal populations, while LMA sponges pump and filter seawater at higher rates (Weisz et al., 2008). HMA sponges have denser mesohyls and complex aquiferous systems with smaller choanocyte chambers, which allow for slower filtration rates (ranging from 52 to 94 % the filtration capacity of LMA sponges) and greater surface area for seawater recirculation (Vacelet and Donadey, 1977; Boury-Esnault and Vacelet, 1990; Weisz et al., 2008). This optimizes feeding on particulate (POM) and dissolved (DOM) organic matter, and inorganic material exchanges (Vogel, 1994), while creating internal microenvironments that foster microbial diversification and functional redundancy (Weisz et al., 2008; Tilman, 1999; Loreau, 2000; Konopka, 2009; Konopka et al., 2015). Therefore, HMA sponges that harbour richer microbiota diversities are likely to be less affected by OA, due to higher chances of compensatory dynamics among functionally similar stains, facilitating stress-resistance and network complexity (Lesser et al., 2022; Sabrina Pankey et al., 2022).

Under changing environmental conditions such as OA, microbiome dynamics may contribute to transgenerational acclimatization (Webster and Reusch, 2017), involving microbial frequency shifts of current strains (symbiont shuffling), introduction of new taxa and/or depletion of extant taxa (symbiont switching) and/or horizontal gene transfer between strains. Preservation of beneficial symbionts is often correlated with host well-being, and stress tolerance. Nonetheless, new microbial acquisitions from the surrounding environment can foment functional stability to withstand future OA, by combining the original partners with locally adapted taxa (Morrow et al., 2015; Ribes et al., 2016). Still, these

statements remain approximative, and pending empirical proof, since metagenomics approaches monitoring microbiome dynamics do not unravel the actual beneficial nature of associated strains. Certain community measurements, though, including changes in alpha and/or beta diversity, or microbial sharing with environmental samples, are commonly used to determine the health or dysbiotic status of symbiotic communities (Luter et al., 2012; Simister et al., 2012; Halfvarson et al., 2017; Lloyd-Price et al., 2019).

The term 'core microbiome' has received increasing attention among microbial ecologists over the last decade (Neu et al., 2021). Determined by stable, consistent microbial taxa found across a defined environment, within one or several hosts species, these core beneficial symbionts are critical for ecological and physiological competence (Hartman et al., 2009; Cho and Blaser, 2012; Lozupone et al., 2012; Relman, 2012; Shade and Handelsman, 2012; Missaghi et al., 2014). However, especially for filter-feeders, microbially diverse cores seem to boost microbiome stability, and confer resistance against the establishment of opportunistic taxa that sponges are constantly encountering through their filtering activities (Björk et al., 2018). Core members are often resilient under changing conditions (Kokou et al., 2018), and this is thought to be due to genetic versatility, attained by large genomes and/or horizontal gene transfer (Thomas et al., 2010; Wiedenbeck and Cohan, 2011; Fan et al., 2012; Gao et al., 2014; Horn et al., 2016; Pita et al., 2018). Furthermore, environmental bacterial DNA, can incorporate mobile elements into the symbiotic genetic repertoire, transferring novel functions without changes in taxonomical compositions (Rosenberg et al., 2007; Sobczyk and Hazen, 2009; Wiedenbeck and Cohan, 2011; Putnam et al., 2017). All things considered, the examination of core microbiome dynamics and composition within sponges thriving under OA, can disentangle the mechanisms of core taxa versatility, in correlation with essential putative functions that support host resilience under environmental challenges.

Underwater volcanic CO₂ vents represent optimal natural laboratories to study long-term effects of OA on sponge holobionts (Fabricius et al., 2015; Fabricius et al., 2011; Goodwin et al., 2014; Uthicke et al., 2013). The number of vent systems worldwide suitable for this kind of researches is limited, including those located around Ischia Island, Columbretes, Faial in the Azores, and Papua New Guinea (González-Delgado and Hernández, 2018; Aiuppa et al., 2021). Among these, the CO₂ vent systems along the Castello Aragonese inlet off Ischia Island are widely utilized for in situ OA experimentation (Hall-Spencer et al., 2008; Kroeker et al., 2011, 2013; Foo et al., 2019), offering low pH zones with a decrease in surface pH from -0.14 to -0.4 pH units (relative to 1870), and applicable for future predictions of year 2100 (Bopp et al., 2013; Gattuso et al., 2015). Mean pH values of these systems range between 7.2 and 7.8 (Teixidó et al., 2018). Recently, a new CO₂ vent system has been discovered along the coast of Ischia. This system includes a semi-submerged cave, where seawater is naturally acidified with pHT ranging from ~7.65 to 7.88 (pH on total scale) (Teixidó et al., 2020).

In this study, we investigated the microbial communities associated with natural sponge assemblages inhabiting two semi-submerged caves: one containing CO₂ vents, providing naturally acidified OA conditions, and another one with no volcanic activity serving as control off Ischia Island. We focused on two Mediterranean demosponge species, an HMA species *Chondrosia reniformis* and an LMA sponge *Spirastrella cunctatrix*. Compositional shifts and presence/absence of microbial Amplicon Sequence Variants (ASVs) were examined, to estimate whether these hosts experience symbiont shuffling and/or symbiont switching, in response to the different natural pH conditions. The 16S rRNA marker gene was utilized to characterize the prokaryotic communities, along with downstream bioinformatic tools that assessed microbial diversities, dysbiosis scores, core microbiome fluctuations, and identified differentially abundant taxa associated with vent and control hosts. Our goal was to compare sponge microbiome structures in neighboring acclimatized host populations, living under ambient and lowered pH conditions, in order to understand how microbial symbionts may assist these holobionts to withstand OA.

2. Material and methods

2.1. Study sites and carbonate chemistry

Here we compare natural populations of the sponges *Chondrosia reniformis* and *Spirastrella cunctatrix* at a volcanic CO₂ vent site and a control site with Ambient pH along the coast of Ischia, in the Gulf of Naples (Italy) (Fig. 1a). The CO₂ vent system is located at 5 m depth inside a semi-submerged cave named Grotta del Mago (40°42'41.87"N, 13°57'51.06"E; hereafter 'vent', and CO₂ gas bubbles emerge from the seafloor at 5 m depth). The CO₂ vent systems locally acidify the seawater with gas comprising 92–95 % CO₂ and do not elevate temperature (Supplementary Fig. S1). The control site with ambient pH named Punta Vico (40°45'32.28"N, 13°52'55.38"E; hereafter 'control') was chosen following the criteria: a) the two species naturally occurred, b) it is also a semi-submerged cave with same depth of seafloor (5 m) and light exposure (Supplementary Fig. S2), and c) no venting activity was evident. The two target sponges species are abundant between 1 m and 2 m depths at both study sites. The two study sites experienced similar seawater temperature conditions, which followed ambient seasonal fluctuations from 15 °C in winter and from 26 to 27 °C in summer (Supplementary Fig. S1, Table S1). Dissolved inorganic nutrients (nitrogen, phosphate, and silicate) showed similar concentrations at both study sites (Supplementary Fig. S3). SeaFET™ Ocean pH sensors (Satlantic) were deployed to quantify variation in pH at the CO₂ vent and the ambient pH sites. Measurements were taken every 15 min. One sensor was deployed at the vent site for 20 days at 2 m depth, and one sensor was deployed for 15 days at the Ambient pH site. The carbonate chemistry derived from discrete water samples and in situ monitoring of seawater pHT (pH on the total scale) revealed a decrease in mean pHT at the CO₂ vent system associated with increased variability in pHT caused by the venting (Fig. 1B). Mean pHT was (mean with 25th and 75th percentiles): 7.65 (7.58, 7.90) with $n = 1530$ measurements for the Vent and 8.05 (8.03, 8.07), $n = 1331$ measurements for the Ambient pH site (see Table S1 for detailed carbonate system parameters). Reductions in seawater pHT at the vent were driven by higher pCO₂ concentrations at relatively constant total alkalinities (mean AT, Vent = 2564 μmol/Kg versus Ambient = 2618 μmol/Kg) and dissolved inorganic carbon concentrations (mean CT, Vent = 2542 μmol/Kg versus Ambient = 2262 μmol/K) (Table S2). Mean pCO₂ was 2905 ± 1664 μatm at the Vent and 322 ± 34 μatm at the ambient pH site (Table S2). The saturation states of aragonite (Ω_a) and calcite (Ω_c) were consistently higher than 1 at the CO₂ vent and higher than 3 at the ambient pH site (Table S1). For full details of pH sensors deployment, calibration, and total alkalinity standard operating protocols, see Teixidó et al. (2020).

2.2. Sponge sampling and experimental design

Fragments ($n = 40$, size ~3.5 cm³) of *C. reniformis* and *S. cunctatrix* were collected in both study sites in July 2021 by scuba diving at 2–4 m depth ($n = 10$ specimens per species and site). Sponges were sampled individually and transported in separate zip bags in coolers to the laboratory, where sub-samples (<0.5 cm³) were rinsed with sterile seawater, snap-frozen in liquid nitrogen and stored at -80 °C until analyzed. Environmental seawater was collected in triplicate (5 L, $n = 3$) at each site near the sponge assemblages. Volumes of 1 L per seawater sample were filtered through 0.22 μm pore size filters (Millipore MF™-Membrane), and filters were stored at -80 °C until processing.

2.3. DNA extraction, library construction, and sequencing

DNA from frozen sponge samples and seawater filters was extracted using QIAGEN PowerSoil Pro Kit (2018), following manufacturer's instructions. Quantification and quality control of the extracted DNA was assessed through a Thermo Scientific Nanodrop™ 1000. Aliquots of the extracted DNA were sent to the Station Biologique de Roscoff (Platform

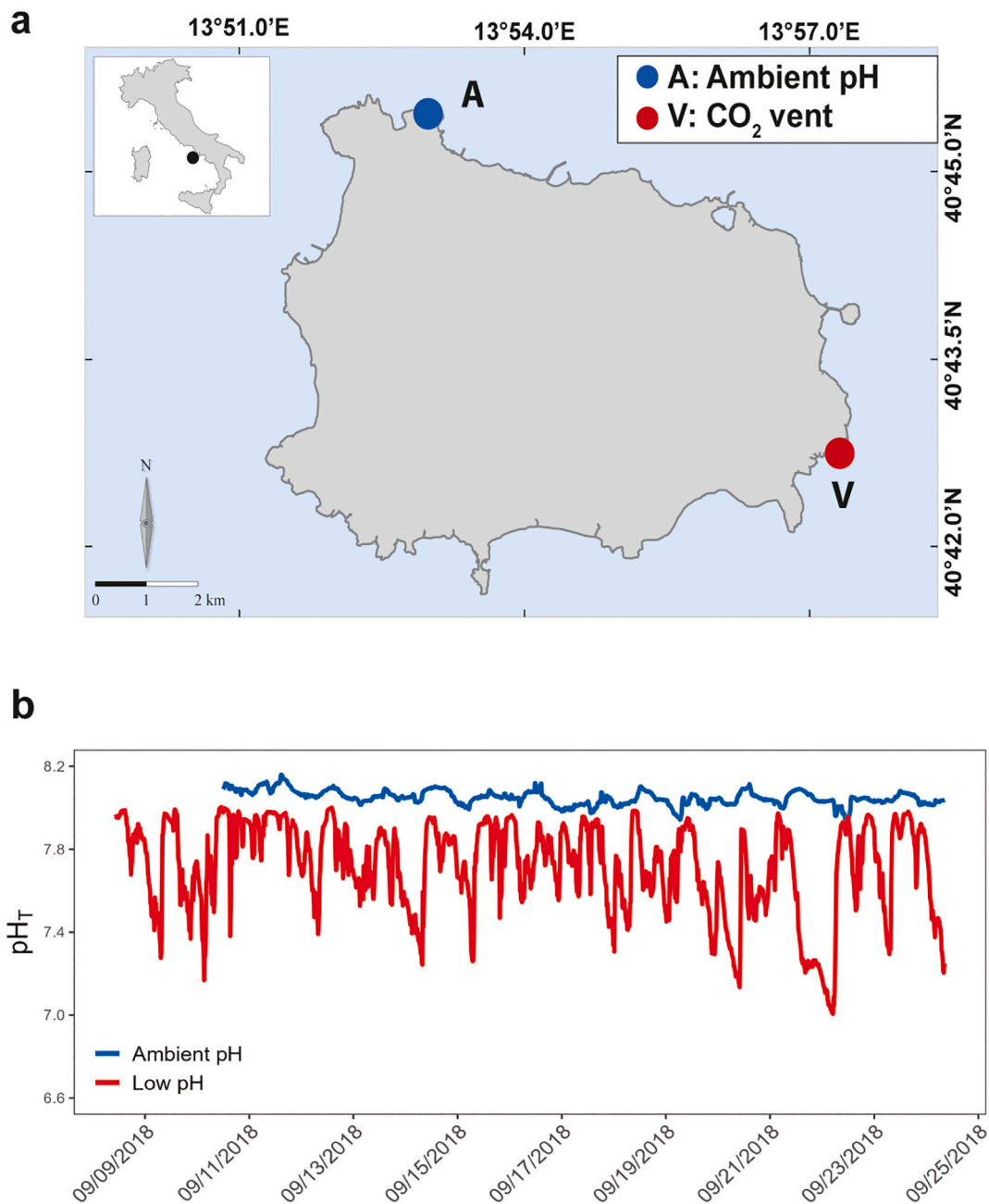


Fig. 1. Location of the study area in Ischia Island and pH time series. A) The CO₂ vent site Grotta del Mago (V, red point) has underwater volcanic CO₂ vents that naturally acidified the seawater and the site Grotta Punta Vico (A, blue point) is an off-vent control site with ambient pH. B) Time series of pH_T (on total scale) at the CO₂ vent site (red) and ambient site (blue) at 2 m depth. Measurements were taken every 15 min using SeaFETs pH sensors for a period of ~15–20 days. Number of measurements = 1530 at the CO₂ vent site and $n = 1331$ for the ambient pH site.

Genomer; SBR France) for amplicon library preparations and sequencing. The 16S V₃-V₄ locus (position 341–805 of *Escherichia coli*) was amplified using universal bacterial and archaeal primer pairs (Bakt_341F and Bakt_805R), and including Illumina adapters (forward primer: TCGTCCGACGCGTCAGATGTGTATAAGAGACAGCTACGGGNGGCWGCAGGTCTCGTGG and reverse primer: GCTCGGAGATGTGTA-TAAGAGACAGGACTACHVGGGTATCTAATCC; (Klindworth et al., 2013). Then a standard Illumina protocol for metabarcoding (Illumina Inc, 2013) for Illumina MiSeq Systems (Wasimuddin et al., 2020; Fadeev et al., 2021) was run using the Q5® High-Fidelity PCR Kit (New England BioLabs, MA, USA), the AMPure XP for PCR Purification Kit (Beckman Coulter, Brea, CA, USA) and the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA). Libraries were quantified with a Quantifluor® ds DNA System (Promega, WI, USA) and mean fragment size was determined using a LabChip® GX Touch™ (Perkin Elmer, MA, USA). An equimolar pool of all samples was generated at a concentration of 4 nM, diluted to 4 pM, spiked with 10 % PhiX (Illumina) and sequenced on an Illumina MiSeq sequencer at the Genomer platform (Station Biologique de Roscoff) using a MiSeq v3 kit (2x300bp, paired-end, 600-cycle) to target ~400–450 bp amplification products.

2.4. Bioinformatic analysis

Demultiplexed 16S rDNA gene amplicon sequence data from 40 sponge and 6 seawater samples were loaded into QIIME2 environment v.2021.11 (Bolyen et al., 2019), and raw reads were assembled and denoised using DADA2 (Callahan et al., 2016) to obtain “Amplicon Sequence Variants” (ASVs) feature frequency table. Taxonomy was assigned against the 16S SILVA (99 % identity) v.138.1 reference database (Quast et al., 2013), which was pre-trained using the QIIME2 RESCRIPt plugin, and evaluated with the evaluate-fit-classifier command (Robeson et al., 2021). Sequencing yielded a total of 346,123 reads and 1676 ASVs after DADA2 pre-processing steps for high quality sequences. Filtering steps to remove singletons, low prevalence (< 10 % across all samples) ASVs and sequences matching mitochondrion and chloroplast resulted in a clean feature table with 319,408 total reads and 390 ASVs across 46 samples (ranging from 1173 to 46,909 reads per sample). The rarefaction of this feature table at a sampling depth of 1173 (reads) yielded 319,408 reads and 385 ASVs, keeping all original samples (Supplementary Tables S3 and S4).

Based on the rarefied ASV tables, we calculated four different parameters for community composition, including 1) Alpha diversity, 2) Beta diversity, 3) Relative abundances, and 4) Beta dispersion and Dysbiosis estimations. Four analytical approaches were instead calculated from unrarefied ASV tables, including 1) Shared microbial ASVs, 2) Core Microbiome (size and composition), 3) Compositional (beta) diversity drivers, and 4) Differentially abundant microbes. Finally, assigned functions on most relevant differentially abundant taxa were identified using exhaustive bibliography searchers. Downstream analyses and statistics followed a bioinformatic workflow as shown in Supplementary Fig. S4 QIIME2R package was used to import QIIME2 artifacts into the R environment as R objects (<https://github.com/jbisan/z/qiime2R>), while phyloseq (McMurdie and Holmes, 2013), vegan (Oksanen et al., 2008), ggplot2 (Wickham, 2016) were used for downstream statistics and plotting.

2.4.1. Community composition

Alpha diversity (within samples) and beta diversity (between samples, see below) matrices were computed with classical relative abundance based methods, on rarefied feature tables on QIIME2 (Bolyen et al., 2019) applying q2-core-diversity-phylogenetics. This command performs a prior normalization to relative abundance, and then calculates several alpha and beta diversity measures. Observed ASVs, and Shannon alpha diversity index were selected for boxplots representation using ggplot2 (Wickham, 2016). Statistical testing was computed using pairwise Kruskal–Wallis Tests to reveal differences between sample

groups ($p < 0.05$) and pairwise Kruskal–Wallis tests with Benjamini–Hochberg false discovery rate (FDR) corrections for multiple comparisons ($q < 0.05$; i.e., FDR adjusted p value).

For beta diversity analyses unweighted UniFrac matrices from the q2-core-diversity-phylogenetics command were selected for visualization in PCoA plots and community analyses. Permutational analysis of variance (PERMANOVA, Adonis) and pairwise analysis of similarity (Anosim) were computed to evaluate changes in microbiome structure and composition per species across sites. All significance thresholds for statistical tests were set at $p < 0.05$ ($q < 0.05$; i.e., FDR adjusted p value).

Variation in community structure (inter individual intra group variability) was determined with beta dispersion analysis (permdisp), which measures the average dissimilarity from individual observation units (here: ASVs) to their group centroid in a multivariate space (Anderson et al., 2006). Permdisp tests were set to 999 permutations and significance to $q < 0.05$ (i.e., FDR adjusted p value).

Dysbiosis estimations were computed with the R package dysbiosisR (Shetty et al., 2022) for each holobiont species across sites using three measures, and setting control site sponges as reference group. Dysbiosis Score of median community level variation – CLV (Lloyd-Price et al., 2019) and distance to reference plane – DRP (Halfvarson et al., 2017), were based on Unweighted Unifrac distances; whereas Symptom or Dysbiosis Index is a on Random forest and out of bag (OOB) predicted probability approach (Saffouri et al., 2019). ROC (Receiver Operating Characteristics) probability curves were computed for all measures, and AUC (Area Under The Curve) values >0.75 determined acceptable separability between dysbiotic and normobiotic classification states (Bradley, 1997). Results were plotted in gradient plots with ggplot2.

Microbial community composition in relative abundances at different taxonomic levels were based on rarefied data, and were visualized in stacked bar plots grouped per metadata categories, i.e. species and site.

2.4.2. Core microbiome

Core microbiome analyses were performed on unrarefied data, and core ASVs were defined at 100 % of prevalence (present in all replicates) across one environment: control site ($n = 10$ sponges, $n = 3$ seawater samples), vent site ($n = 10$ sponges, $n = 3$ seawater samples) or both sites (control+vent; $n = 20$ sponges, $n = 6$ seawater samples). Bullseye plots were generated for the representation of core sizes and pie plots for the core microbiome composition. Core microbiome was calculated in both sponge holobionts for functional interpretations in the context of enclosed biological symbiotic systems; whereas in seawater samples these analyses were computed for descriptive purposes, for taking part of an environmental entity non-biologically enclosed.

2.4.3. Microbial compositional analyses

Compositional data analyses aim at identifying differentially abundant taxa driving sample grouping. It compares ratios (log-fold changes) of each feature across metadata categories, while removing the biases of missing the total microbial load information.

2.4.3.1. Compositional diversity drivers. Compositional beta diversity based on Aitchison distances was calculated using QIIME2 DEICODE plugin (<https://github.com/biocore/DEICODE>) on non-rarefied data, applying matrix completion (to solve the ‘zero-problem’ and high levels of sparsity (Keshavan et al., n.d.)). For Aitchison distance, counts data was centered log transformed (CLR), and then the Euclidean distance was computed with a non-supervised method (no covariates are introduced in the formula). The resulting matrix was used as input for robust principal-component analysis (RPCA) biplots, in which ASVs responsible for beta-diversity shifts were visualized as vectors in the ordination (Martino et al., 2019).

Log ratios of the microbial balance formed by the most defining vectors/ASVs driving differences in the ordination space were visualized

with Qurro (Fedarko et al., 2020) (<https://github.com/biocore/qurro>; see below). Statistical differences of the resulting log ratios between different sample groups, i.e. sponge species or seawater from vent versus control site, were determined using the Welch's Heteroscedastic F Test ($p < 0.05$).

2.4.3.2. Differentially abundant microbes. Songbird (v1.0.3; <https://github.com/biocore/songbird>) was used to identify differentially abundant features (ASVs) across site, by determining the log-fold changes of ASVs within *C. reniformis* and *S. cunctatrix*, associated to either the control or the vent site (Morton et al., 2019). Unrarefied feature tables were fed separately for each sponge species to eliminate 'species' as a covariate and focus solely on the effect of 'site'. Briefly, Songbird uses a multinomial regression model to calculate differential ranks, implemented using TensorFlow (Abadi et al., 2016). Two models were created separately for *C. reniformis* and *S. cunctatrix*. For each sponge species 'site' was added as covariate in the formula of the linear model (supervised method). Overfitting and accuracy of each generated model were determined by comparing against a null model ('1' in formula). A pseudo Q^2 value close to 1 indicated a high predictive accuracy, as opposed to a pseudo Q^2 close or below zero, representing poor predictive accuracy.

Microbial balances were generated for each sponge species to separate sample groups (control versus vent specimens), with features sorted according to their log-fold change as numerator or denominator taxa, in correlation to a metadata co-variate category –in our case “site”. Microbial balances were visualized as boxplots through 'Qurro' (<https://github.com/biocore/qurro>), where the selected features were grouped across sample groups yielding differential ranks values (log ratios). Higher log-ratios in a sample group stand for positive correlation with numerator features and, consequently negative ranking with denominator features (e.g. if samples from the vent site had higher log-ratios, then these samples were associated to an increased abundance of the numerator taxa and/or lower abundance of denominator taxa); and vice versa, lower log-ratios denote positive ranking with denominator features and negative correlation with numerator taxa (e.g. if samples from control site had lower log-ratios, then these were more abundant in denominator features and/or less abundant in numerator taxa). Statistical differences of the log ratios resulting from selected numerator and denominator taxa were determined using the Welch's Heteroscedastic F Test ($p < 0.05$).

2.4.4. Assigned functions of key differential microbes

Putative assigned functions of key differentially abundant microbes were based on literature surveys. We identified 19 key ASVs that were significantly correlated with either acidified vent or control sites in the respective sponge host species. For this consortium of microbial drivers we described the associated functions and interpretations, in the context of topic published work on marine sponge ecology, acclimatization and physiology (Supplementary Table 3).

3. Results and discussion

3.1. Sponge morphology and microbial diversity changed in response to OA

3.1.1. Remarkable body surface reduction characterized *S. cunctatrix* vent populations

Conspicuous morphological differences were observed for *S. cunctatrix* at the vent site. The sponges showed rachitic growth appearance, with evident tissue shrinkage/thinning, reduced body surface area, narrower water canals and smaller oscula (Fig. 2a). Diameter of the oscula ranged in control conditions between 3 and 5 mm, whereas at the vent site maximum oscula size was ≤ 1 mm. *Chondrosia reniformis* specimens instead, had similar massive body volume and oscula sizes

across both sites (Fig. 3b). Interestingly, a similar response has been observed in the coral *Astroides calycularis*, which also naturally inhabits the CO₂ vent site. Colonies of this coral were weaker, smaller, and exhibited a thinner or absent coenosarc (the tissue covering the skeleton located between the polyps) (Teixidó et al., 2020). These changes in morphological traits may suggest similar stress responses to acidification, and could indicate shifts in species traits to persist in these high pCO₂ habitats.

3.1.2. Increased alpha diversity in *S. cunctatrix* under vent conditions

Alpha diversity across CO₂ vent and control sites revealed different trends in both sponges holobionts and the seawater. Observed ASVs, as the number of different ASVs present in a sample, and Shannon index were significantly lower for *C. reniformis* in the vent site compared to control samples. Instead, vent *S. cunctatrix* specimens displayed significantly higher Shannon index with respect to the corresponding controls, linked with enhanced microbial diversity and richness in the vent seawater (Kruskal-Wallis, $q < 0.05$; Tables S5, S6; Fig. 2c,d). Increases in (gamma- and alphaproteo-) bacterial diversity in thermally stressed *Lanthella basta* were proposed to be caused by a breakdown in antimicrobial compound production preceding sponge mortality, hence allowing the proliferation of opportunistic bacteria (Luter et al., 2012). Accordingly, increased Shannon diversity in vent *S. cunctatrix* sponges could be a first indicator for stress-induced dysbiosis in the symbiotic community. This higher alpha diversity trend in *S. cunctatrix* linked to that of the vent seawater (as opposed to *C. reniformis*) could be related to the larger filtering activities of LMA sponges in comparison to HMA sponges (Weisz et al., 2008; Ludeman et al., 2017; Morganti et al., 2017). Thus, *S. cunctatrix* may acquire more microbial cells from the environment, including opportunist strains (Frank, 1996; Ribes et al., 2015; Oliveira et al., 2020) that contribute to boost richness in concomitance with the water column's microbial loads. While *C. reniformis* may be able to better control the entrance of new strains by maintaining a rich and diversified microbiome (Mazzella et al., unpublished).

3.1.3. Beta diversity showed dissimilarities in both host sponge species across sites

Both sponge species and seawater had different microbial community compositions (PERMANOVA, $q < 0.05$; Table. S6). Comparing samples from vent versus control sites, best clustering was obtained using unweighted UniFrac distances (Fig. 2e; Fig. S5), a qualitative beta diversity distance, which takes into account phylogenetic diversity and emphasizes rare species (Lozupone et al., 2011). The PCoA ordination resolved six clusters, showing clear separation between the two sponges and the seawater, as well as samples distinction for either sponge species from control and vent sites (Fig. 2e; Fig. S5). PERMANOVA pairwise comparisons confirmed the previous clustering for either sponge species between sites, supporting dissimilar community structures in conspecific hosts living under different pH conditions ($q < 0.01$; Table. S8). Instead, seawater samples from control and vent sites yielded non-significant differences in beta diversity ($q > 0.05$; Table. S8).

3.1.4. Microbiome community composition changes under OA

OA conditions induced microbial compositional differences in both sponge species, at the vent site, at all taxonomy levels: from phylum to genus. In the seawater, modest dissimilarities, yet not significant, could be also detected across sites (Supplemental Fig. S5, S6 and detailed results therein). In Fig. 2f, the class level prokaryotic community is selected, primarily for its informative potential on predicted functionalities.

In *C. reniformis*, the major phylum, Proteobacteria increased at the vent site, mainly due to increases of Gammaproteobacteria, whereas other prevalent phyla like Chloroflexi, Poribacteria and Acidobacteria, decreased their relative abundances at the vent. The microbiome of *S. cunctatrix* instead, was dominated by Alpha- and

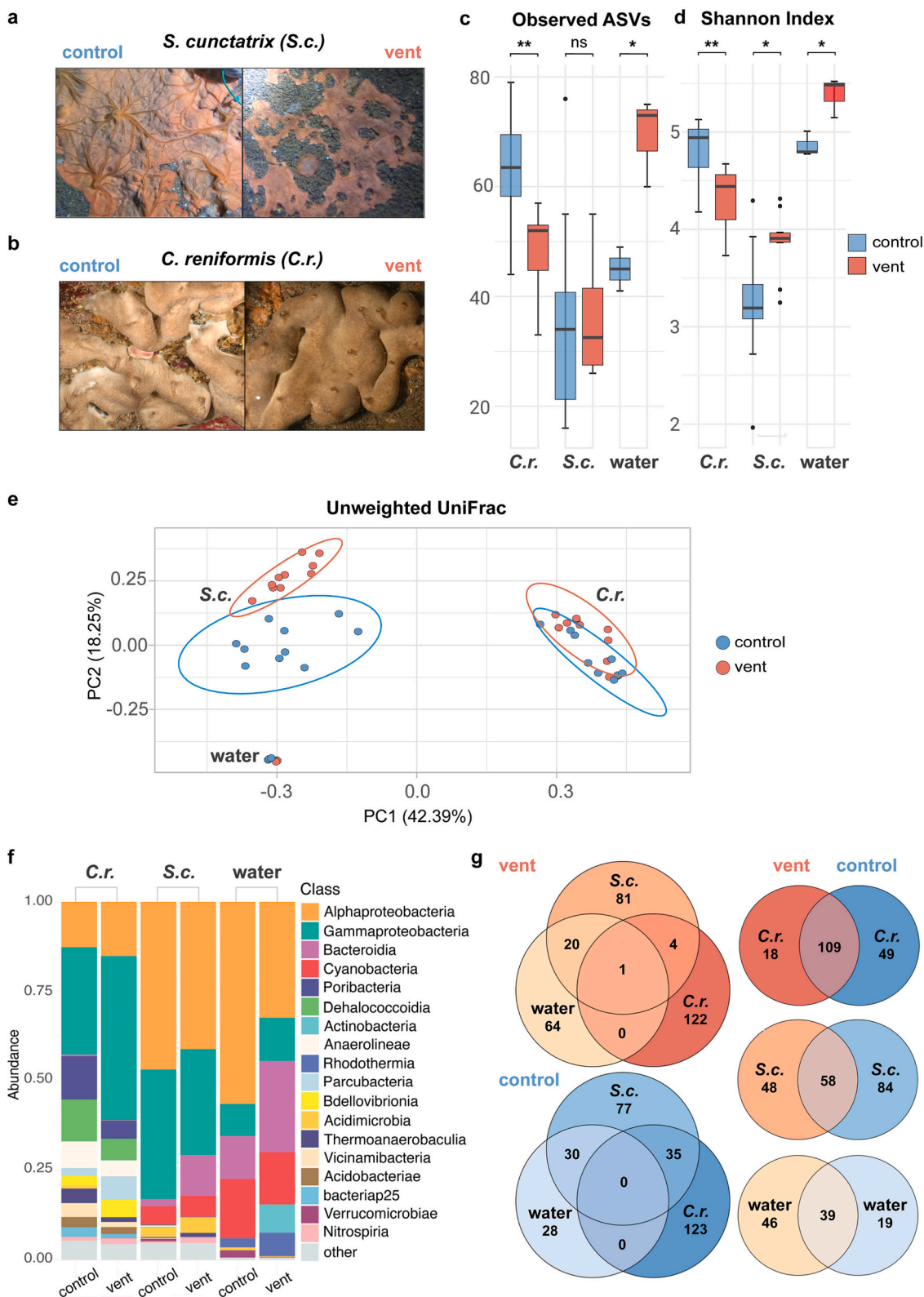


Fig. 2. Sponge morphology and bacterial diversity in *C. reniformis* and *S. cunctatrix* sponges in in situ vent and control conditions. a) *S. cunctatrix* sponges' morphology showed tissue thinning, reduced body surface and smaller oscula and channels in vent compared to control conditions. b) *C. reniformis* sponges showed similar morphology across sites. c) Observed ASVs and d) Shannon index for sponge and seawater samples from control and vent sites. e) PCoA based on unweighted UniFrac beta diversity showed separate clusters for both sponge species across sites (Pairwise Anosim; $q < 0.01$), and non-significant clustering by site in water samples (Pairwise Anosim; $q > 0.05$), ordinations with other PC axes are provided in Fig. S5.. f) Taxa bar plots showing microbial relative abundances for *C. reniformis*, *S. cunctatrix* and seawater samples from the vent and control site ($n = 10$ each); best taxonomic annotation is presented for the most abundant prokaryotic classes. g) Venn Diagrams of shared microbial taxa between *C. reniformis* and *S. cunctatrix* sponges and the seawater from control pH (left) and CO₂ vent site (right). * Shared core microbes, i.e. ASVs present in all 10 sponge replicates per site. This analysis was based on non-rarefied feature tables.

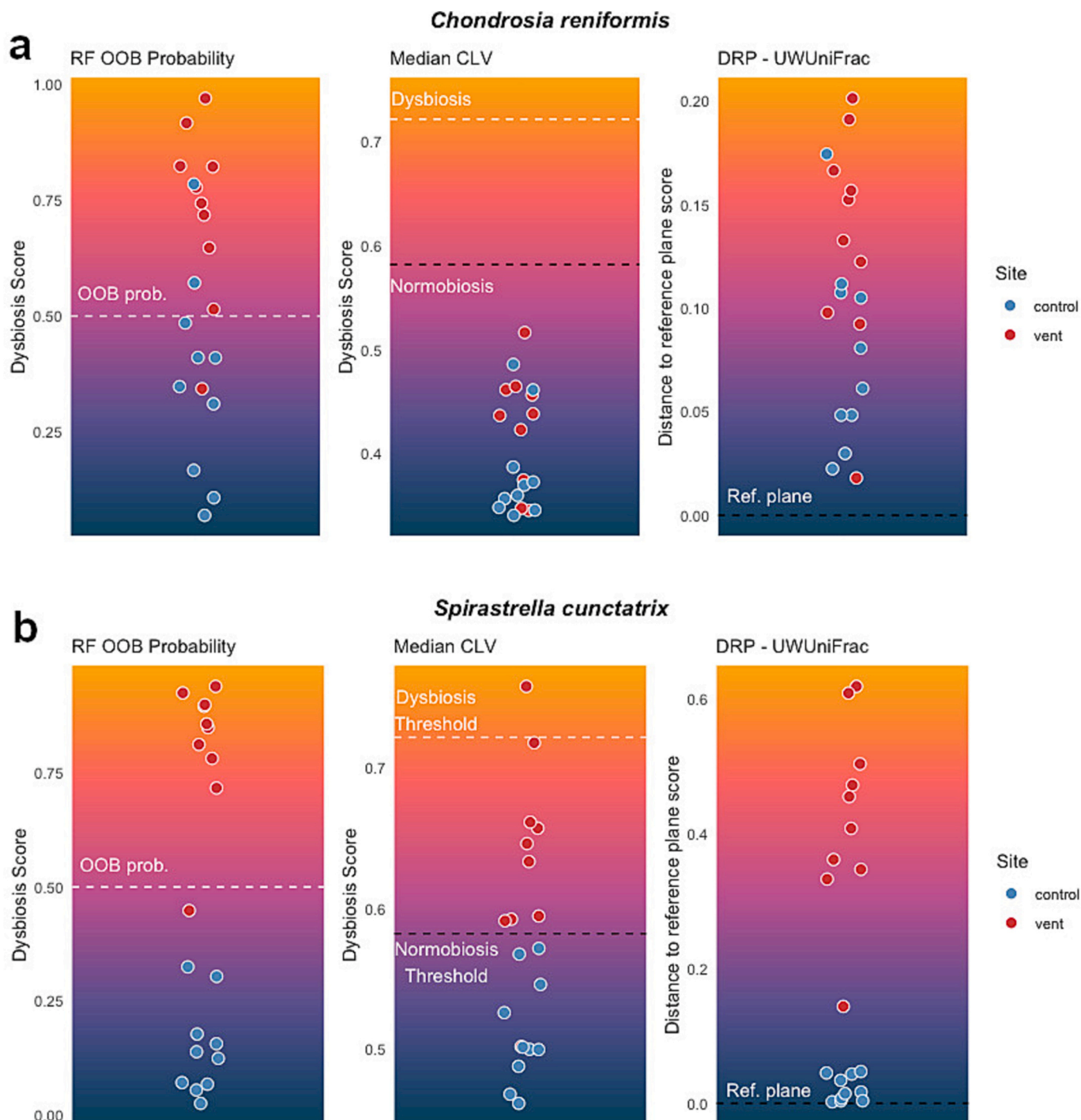


Fig. 3. Gradient plots depicting dysbiosis measures estimations. Scores were based on Random Forest classification and out of bag (OOB) predicted probability for Symptom or Dysbiosis Index (left), MCV – median community level variation (middle), and DRP – Distance to reference plane (right) based on UniFrac distances in *Chondrosia reniformis* (a) and *Spirastrella cunctatrix* (b) sponge holobionts from control site (blue) and vent (red) populations, where control samples are taken as reference group. Dysbiosis scores on OOB probability over 0.75 reflect good probability of the samples being classified as dysbiotic. Normobiotic and Dysbiotic thresholds in median CLV approach are set according to high divergent samples using 10th and 90th percentile of the dysbiosis score respectively, and samples in between these values are in incipient dysbiotic status. Individuals more distant to the Reference plane and exiting the cloud circumscribed by the reference group in the DRP approach have more probability of being classified as dysbiotic.

Gammaproteobacteria, both in lower relative abundance in vent populations with respect to the control. Interestingly, microbial shifts reported in *S. cunctatrix* were mirrored in the microbial communities of the seawater. These included decreases in Proteobacteria from control to vent samples, mainly driven by Alphaproteobacteria, or increases of phylum Bacteroidota and Actinobacteriota. But compared to

S. cunctatrix, Gammaproteobacteria were generally less abundant in the seawater and Cyanobacteria more abundant (see Fig. S6 and Supplementary Microbiome community composition results for details).

OA experiments in aquaria described no microbial changes in the barrel sponge, *Xestospongia muta* (Lesser et al., 2016). Instead, explorations on two tropical sponges –*Coelocarteria singaporensis* (HMA) and

Stylissa cf. flabelliformis (LMA)– living in a control pH reef and an adjacent CO₂ vent in Papua New Guinea revealed disparate outcomes. While the microbial composition of *C. singaporensis* differed significantly between sites, the microbiome of *S. cf. flabelliformis* was rather conserved (Kandler et al., 2018; Morrow et al., 2015). Notwithstanding the seeming divergent trends reported in that study for the LMA and HMA counterparts, certain taxa shifts showed analogies with our sponges. Phyla Chloroflexi and Acidobacteria decreased and Proteobacteria increased in *C. reniformis* living under OA conditions, as well as in the HMA *C. singaporensis* after transplantation on a CO₂ vent area (Kandler et al., 2018; de Menezes et al., 2022; Waterworth et al., 2021). Furthermore, Proteobacteria decreased and Bacteroidota increased in vent *S. cunctatrix* specimens, as did in LMA sponges *Stylissa cf. flabelliformis*, when transplanted to the vent at Papua (Glasi et al., 2018; Kandler et al., 2018). All these findings support that prokaryotic community dynamics in response to environmental challenges seem to maintain a host species-specific print, and this prevails beyond the LMA and HMA condition.

3.1.5. Shared microbes between the two sponge species decreased under OA

Venn diagrams were generated to examine shared microbial taxa between both sponge host microbiomes, as well as their association with the surrounding seawater in control and vent conditions (Fig. 2g). In the control site, 158 ASVs were identified in *C. reniformis* and 142 in *S. cunctatrix*, and these numbers decreased for both sponge species under acidified conditions to 127 and 106 ASVs, respectively. Seawater samples exhibited an opposite trend, reporting an increase in ASVs from 58 in control conditions to 85 ASVs at the vent site. *Chondrosia reniformis* shared 109 ASVs between the control and vent sites, while 18 ASVs were exclusive to the vent samples and 49 ASVs were exclusive to the control samples. In contrast, *S. cunctatrix* from the vent and control sites shared only 58 ASVs, with a substantial number of unique ASVs found in both vent (48) and control (84) sponges. This significant incidence of unique ASVs across vent and control holobionts of the same species, implies symbiont switching with the water column, and in particular the introduction of new microbes from either environment.

In the control site, there were 35 overlapping features between both sponges. These features were primarily classified as Alphaproteobacteria (58 %), Bacteroidia (20 %), and Gammaproteobacteria (11 %). However, at the vent site, the number of shared ASVs drastically decreased (by 90 %) to only 4. These ASVs appertained to Alphaproteobacteria, Gammaproteobacteria, Verrucomicrobiae, and Planctomycetes. This striking reduction in shared ASVs suggests a process of stress-induced dysbiosis in *S. cunctatrix*, as the symbiotic community in this host shifted from sponge-enriched microbes (associated with *C. reniformis*) to seawater microbes, likely representing opportunistic and transient stains closely related to free-living organisms (Simister et al., 2012; Pita et al., 2018).

Spirastrella cunctatrix sponges shared 30 ASVs (21 %) with the ambient water at the control site and 20 ASVs (19 %) at the vent. In either site, the shared ASVs between this sponge and seawater were predominantly classified as Alphaproteobacteria, Bacteroidia, Cyanobacteria, and Gammaproteobacteria, listed in decreasing abundances. *Chondrosia reniformis* in lieu, did not share any microbes with the seawater at the control site, and in acidified conditions, it only shared one ASV belonging to genus *Haloferula* (phylum Verrucomicrobiota). A prior study of Erwin et al. (2015) using restriction enzymes already described *S. cunctatrix* as having bacterial similarities with the seawater, but they also found a great amount of shared ASVs between the seawater and both *C. reniformis* and *S. cunctatrix*. Our results, instead, indicate a scarce microbial sharing of *C. reniformis* with ambient seawater, and notably stronger host-specificity, similar to what Ribes et al. (2016) reported in their work.

3.1.6. Vent holobionts were normobiotic in *C. reniformis* and incipiently dysbiotic in *S. cunctatrix*

Beta dispersion (as the variability in microbial community composition) revealed non-significant differences in within-group variability in either *C. reniformis* nor *S. cunctatrix* microbiomes across sites ($q < 0.05$; Table. S9). This implies that the within-group beta dispersion was smaller than the dispersion between groups (i.e., sites); thus, supporting that across control and vent sites the microbiome composition was different, but showed certain stability across individuals inhabiting the same site. Stress-related increases in sponge microbiome beta dispersion have been reported in manipulative experiments (Lesser et al., 2016; Pineda et al., 2017), as well as under natural perturbations (Erwin et al., 2012; Turon et al., 2019), as an indicator of dysbiosis, according to the ‘Anna Karenina principle’. This principle, postulates that intraspecific variability is higher among dysbiotic individuals than within healthy groups, in which this functional variability is considered ‘normobiotic’ (Zaneveld et al., 2017). Nonetheless, the concept is vague and more studies are demonstrating that dysbiosis is manifested by disruption of microbiome networking, rather than changes in beta dispersion (Marasco et al., 2022), and therefore, it should be estimated from several perspectives (Halfvarson et al., 2017; Lloyd-Price et al., 2019; Saffouri et al., 2019). According to Dysbiosis scores of median community level variation, distance to reference plane and Symptom/Dysbiosis Index based on random forest predictions, vent holobionts of *S. cunctatrix* were classified as mostly dysbiotic (AUC > 9). In lieu, *C. reniformis* only reported discriminative probability (AUC = 7.2) in the Symptom Index, being the other estimates uninformative (AUC < 0.6), indicating that control and vent specimens could be overall considered within a normobiotic status (Fig. 4). These results suggest that *S. cunctatrix* could be thriving in an incipient status of microbial dysbiosis under acidified conditions, in correlation with a seeming rachitic, fragile growth, as reported above, and analogous to other inhabitants of this environment (Teixidó et al., 2020).

3.2. Core compositional shifts and holobiont resilience

3.2.1. Core microbiome size

To distinguish between stable and transient bacterial groups under OA, core versus non-core microbes were examined in both sponge species. Core bacterial communities were defined strictly as features consistently present across all sponge replicates within each sample grouping (species, sites). *Chondrosia reniformis* exhibited more core ASVs than *S. cunctatrix* (Fig. 4g; core taxa indicated with *). Under OA the number of core taxa decreased in *C. reniformis*, from 17 core taxa at the control site to 12 at the CO₂ vent site. Conversely, *S. cunctatrix* displayed an increase in core taxa, with four core ASVs displayed at the control site and nine at the vent site. In the environmental water column, the number of core members increased from 32 taxa in the control site to 48 at the vent, with core microbiome sizes representing 87 % and 85 %, respectively in either site. Nineteen core taxa were shared across both sites, representing a core size of 54 %. Only one seawater vent core member in genus *Cyanobium* PCC-6307 was shared as core taxon with one of the sponge holobionts, in particular with *S. cunctatrix* vent populations (Supplementary S7, S8).

The core microbiome size, referring to the relative abundance of the core ASVs with respect to the overall microbial community, revealed that *C. reniformis* exhibited a smaller core size (50 %) compared to *S. cunctatrix* (68 %), regardless of the sampling site (Fig. 4). When analyzing core microbiomes across both sites and all replicates ($n = 20$) per species, core sizes decreased in both hosts. Such decrease was more pronounced in *C. reniformis* hosts, with core sizes decreasing from 50 ± 11 % to 23 ± 10 %, compared to a 10 % drop in *S. cunctatrix*. Lower microbiome core sizes were similarly disclosed in sponge assemblages from polluted zones, in comparison with host populations from well-preserved zones in Vietnam (Turon et al., 2019). In consequence, our data could indicate significant alterations in the composition of the core microbiome communities in response to OA.

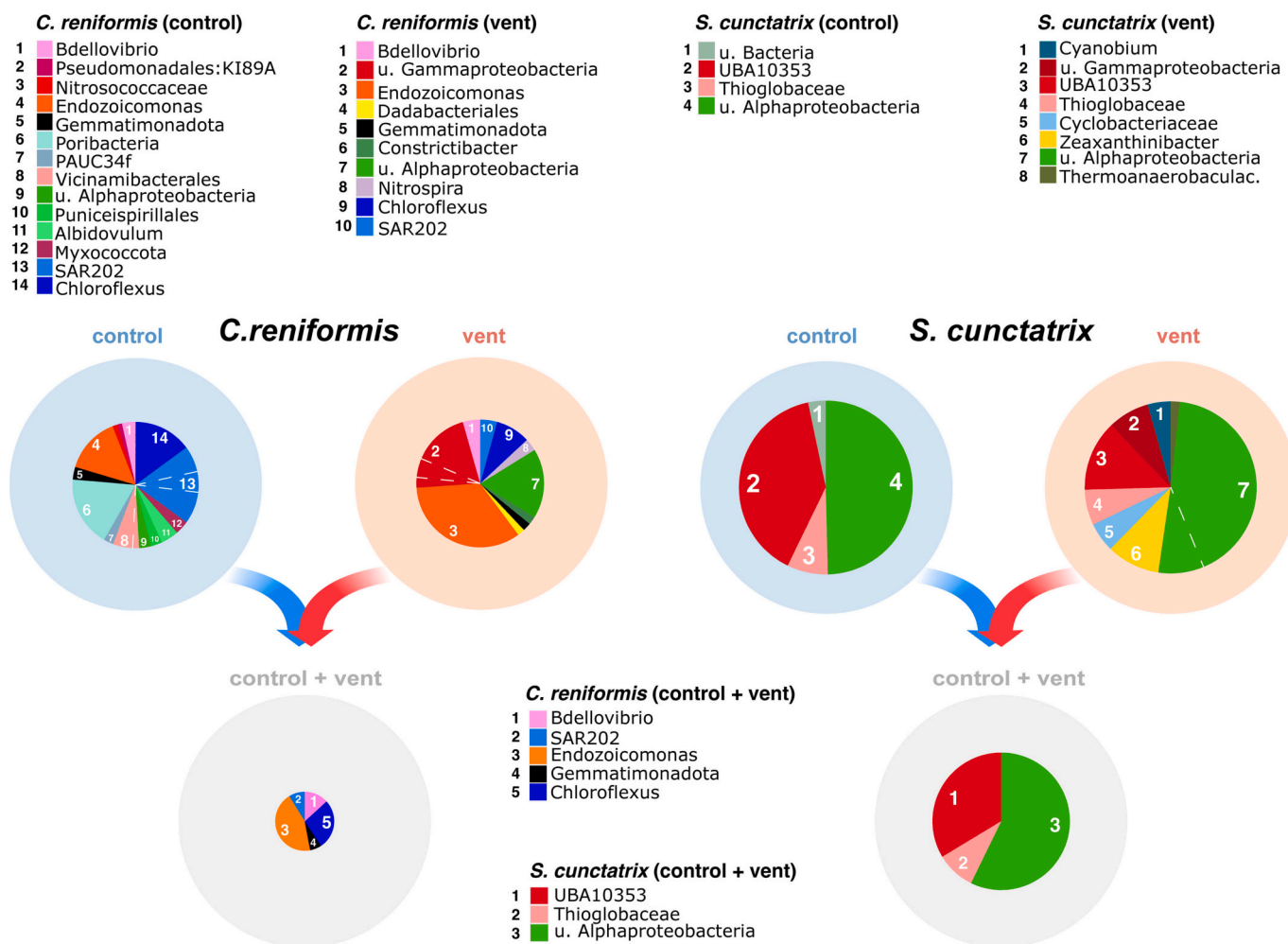


Fig. 4. Core size and core microbiome composition. Core microbiome of *C. reniformis* and *S. cunctatrix* sponges in control (n = 10) and vent (n = 10) sites (both sites, n = 20). Bullseye plots represent the size of the core microbiome relative to the total microbiome. The inner pie charts correspond to the core microbiome and its microbial composition. The outer donut charts represent the variable non-core microbiome. Sky blue donut circles indicate individuals coming from the control site, whereas light pink ones correspond to specimens from the vent site. Taxa names are given at the best annotation level, “U.” stands for “unclassified”.

3.2.2. Core microbiome composition

Chondrosia reniformis exhibited a more diverse and dynamic core microbiome compared to *S. cunctatrix*, displaying notable changes in the relative abundances of the core members in response to OA (Fig. 4). The most prominent core taxa in control conditions were: Poribacteria (18 ± 14 %), an unidentified Chloroflexus (Anaerolineae A4b, 15 ± 8 %), *Endozoicomonas* (14 ± 17 %) and three SAR202 (7 ± 5 % the most represented). Proteobacteria were represented by 11 % of Alphaproteobacteria (Puniceispirillales, Albidovulum and unclassified ASV) and 16 % Gammaproteobacteria (comprised by Pseudomonadales KI89A, Nitrosococcaceae and the already mentioned *Endozoicomonas*). At the vent site Gammaproteobacteria peaked to 55 %, mostly due to *Endozoicomonas* (increasing up to 34 ± 16 %) and three unidentified species. Similarly, the relative core abundance of Alphaproteobacteria increased at the vent site due to a new unclassified ASV (from 11 % to 22 %). Chloroflexi (SAR202, and an unidentified Chloroflexus –Anaerolineae A4b) which comprised 35 % of the core microbiome at the control site, declined to 13 % at the vent site. Two new phyla were introduced into the core microbiome at the vent site: Dadabacteria (order Dadabacteriales; 2 ± 1 %) and Nitrospirota (genus *Nitrospira*; 3 ± 2 %), while 4 phyla were lost: Poribacteria, PAUC34f, two Acidobacteriota (order Vicinamibacteriales) and Myxococcota. Several members of the core microbiota were rare taxa. Shared core ASVs of both sites in *C. reniformis* were dominated by *Endozoicomonas* (44 ± 26 %), followed by

Chloroflexus (Anaerolineae A4b; 27 ± 19 %), Dehalococcoidia (SAR202; 13 ± 9 %), *Bdellovibrio* (9 ± 9 %) and a terrestrial genus BD2–11 (Gemmatimonadota; 6 ± 5 %).

The core microbiome of *S. cunctatrix* from the control site was largely dominated by Alpha- and Gammaproteobacteria, comprising 48 % and 50 %, respectively. Gammaproteobacteria were represented by the dominant genus UBA10353 at 32 ± 14 % and one feature belonging to the family Thioglobaceae at 9 ± 6 %. Interestingly, 6 new features were introduced into the core microbiome at the vent site, one belonging to genus *Zeaxanthinibacter* (Bacteroidota; 10 ± 6 %), one *Cyanobium* (Cyanobacteria; 4 ± 1 %) that was also a core member of the vent seawater (Supplementary Fig. S7, S8), one feature belonging to the family Thermoanaerobaculaceae (Acidobacteria; 2 ± 1 %), one unidentified Cyclobacteriaceae (6 ± 3 %), and two unclassified Alphaproteobacteria and one Gammaproteobacteria. Concomitantly, the relative abundance of core Gammaproteobacteria decreased by 20 % in the vent site, in particular UBA10353 decreased down to 13 ± 8 % (Fig. 4).

The identification of a core microbiome can be useful to classify the health status of microbial communities (Shade and Handelsman, 2012; Cárdenas et al., 2014; Astudillo-García et al., 2017). Indeed, it can be decisive in determining whether the (sponge) holobiont is going through stress-induced dysbiosis or positive acclimatization under challenging conditions, such as OA (Pita et al., 2018). In this study, notable

reduction of core sizes in *C. reniformis* occurred in concomitance with decreases in the number of core taxa, with few overlapping core microbes maintained from control to acidified conditions. In contrast, *S. cunctatrix* showed a minor decrease in core size, and an increased core diversity at the vent site, yet maintaining the major core groups. More diverse and variable core microbiomes have been described in HMA sponges, and in particular in *C. reniformis*; compared to LMA species –including *S. cunctatrix*, which often possess stationary core microbiomes dominated by few key highly abundant taxa (Erwin et al., 2015). Therefore, we propose that *C. reniformis* might display dynamic flexibility for restructuring its core microbiome to adapt to OA (Ribes et al., 2016; Webster and Reusch, 2017; Pita et al., 2018). In this process our *C. reniformis* sponges retained five indispensable taxa, while losing the remaining ten, and introduced six (~60 %) new core groups. As supported by the alpha diversity values (lower in vent specimens), and null microbial sharing with the seawater, new core taxa would have been imported via symbiont shuffling from rare undetectable groups of the variable host microbiome, or other unknown mechanisms (e.g., horizontal gene transfer, or other). In lieu, vent *S. cunctatrix*, retained all but one of the four control core taxa, altogether representing between 90 and 70 % of the core size in either site. The new taxa introductions in *S. cunctatrix* vent cores (six taxa) were acquired likely via symbiont shifting, as supported by the increased alpha diversity values, and large

taxa exchanges with the seawater (even if other intake modes cannot be discarded). Considering that the core microbiome of other LMA sponges is defined as highly steady over time, geography and/or stress pressures (Erwin et al., 2015; Vargas et al., 2021; Happel et al., 2022), these environmental microbial inputs, profoundly reshaping *S. cunctatrix* core microbiome could diagnose a bypass stress rearrangement (dysbiosis) towards OA.

3.3. Identification of differentially abundant microbes associated to OA

Chondrosia reniformis holobionts yielded 46 differentials (differentially abundant ASVs), when selecting the top 20 % most correlated features towards either the vent or the control site, based on supervised multinomial regression using Songbird. For *S. cunctatrix*, 37 differentials were obtained from selecting the top 36 % most significantly informative ASVs according to ‘site’ (vent versus control) co-variate. In either model numerator taxa (red bars) had positive centered log-ratios (log-fold changes), and were associated with the CO₂ vent samples. Whereas denominator ASVs (blue bars) showed negative log-fold changes, and were more abundant in sponges from the control site (Fig. 5a). Notably, a significant number of ASVs belonging to Alphaproteobacteria, Pseudomonadales (SAR86), and Cryomorphaceae demonstrated a strong affiliation with the control site in both sponge species. Additionally, the

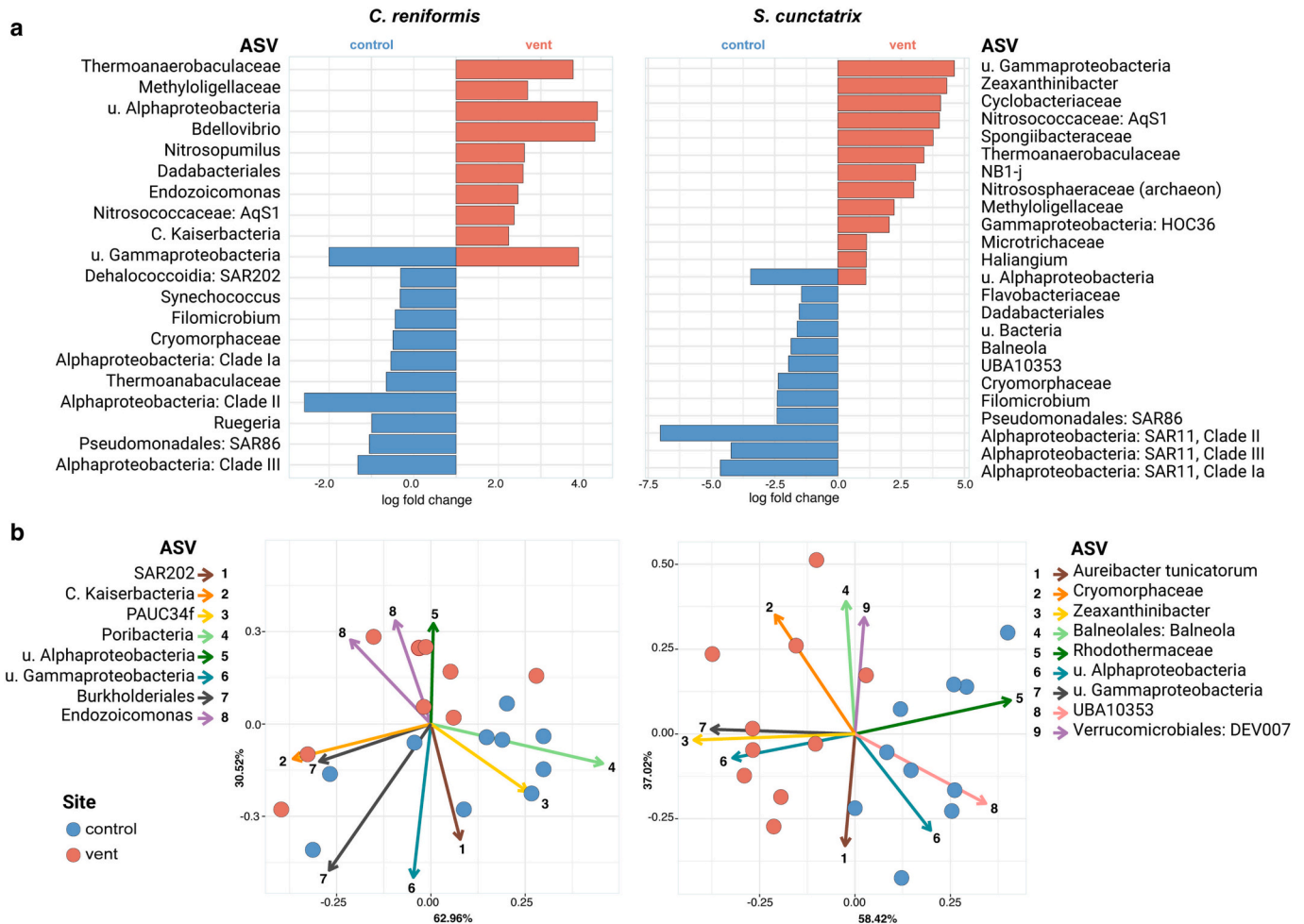


Fig. 5. Differentially abundant ASVs and compositional beta diversity drivers. a) Divergent plots showing the log fold changes of the most informative 20 and 24 differentially abundant ASVs for the *C. reniformis* and *S. cunctatrix* sponges, respectively, ranked according to their association with either control or vent sites. Only differentials with a minimal log fold change of -1 or $+1$ are displayed. b) Compositional DEICODE RPCA biplot of beta diversity from microbial communities associated to *C. reniformis* and *S. cunctatrix* based on Aitchison distances. Circles represent individual samples from control and vent sites. Most relevant taxa driving differences in the ordination space are identified as vectors labeled with the respective taxonomy. Arrows directions indicate association of ASVs towards vent or control host microbiomes.

presence of Nitrosococcaceae (AqS1) was commonly observed at the vent site across both hosts. However, disparate variations were observed in the case of Dadabacteriales, as they exhibited a pronounced preference for vent sites in *C. reniformis*, while in *S. cunctatrix*, they were more closely associated with control sites. Regarding some unclassified Gammaproteobacteria microbes, their distribution in *C. reniformis* showed associations with both vent and control sites, whereas in *S. cunctatrix*, Gammaproteobacteria were predominantly linked to the vent site (Fig. 5a).

The unsupervised DEICODE analyses reported significant differences in compositional beta diversity between the sampling sites within each sponge species (PERMANOVA, $p < 0.05$). Major taxa driving site differences in the RPCA ordination biplots (Fig. 5b) were investigated in taxa-to-taxa relationships, creating microbial balances. In *C. reniformis* ASVs mostly accounting for site grouping separation, yielding significantly different $\frac{\text{numerator}}{\text{denominator}}$ ratios were: $\frac{\text{Bdellovibrio}}{\text{SAR202}}$ (Supplementary Fig. 5), $\frac{\text{Endozoicomonas}}{\text{Burkholderiales}}$ (Supplementary Fig. 6) and $\frac{\text{Endozoicomonas}}{\text{Chloroflexus}}$ (Supplementary Fig. 7). ASVs belonging to the genus *Bdellovibrio* and *Endozoicomonas* were more associated with sponge samples from the vent, while SAR202, Burkholderiales and an unidentified Chloroflexus feature were associated with *C. reniformis* inhabiting the control site. Differentially abundant taxa mostly accounting for beta diversity shifts across sites in *S. cunctatrix* sponges with significantly different $\frac{\text{numerator}}{\text{denominator}}$ ratios were: $\frac{\text{UBA10353}}{\text{Zeaxanthinibacter}}$ (Supplementary Fig. 8), $\frac{\text{UBA10353}}{\text{Pseudomonadales}}$ (Supplementary Fig. 9) and $\frac{\text{SAR11}}{\text{Pseudomonadales}}$ (Supplementary Fig. 10). Genus UBA10353 was associated with the control site, while the genus *Zeaxanthinibacter* and the order Pseudomonadales were associated with the vent site. Strikingly, in all cases these major taxa drivers of either vent or control conditions, were part of the core microbiome of either sponge host.

Differentially abundant core symbionts driving separation between low pH and control pH adapted holobionts represent a promising avenue for understanding the role of microbes in the response of sponges towards OA. Investigating their potential functional regulatory roles and ecological versatility can shed light on the mechanisms underlying the sponges' ability to adapt and thrive in changing ocean conditions (Fig. 6). Box 1 highlights the potential significance of core symbiont taxa in holobiont stress responses towards OA, acclimatization and adaptations processes and fitness.

Our results suggest that OA promoted the prevalence of core microbes involved in the carbon-, nitrogen and sulfur-metabolisms for biogeochemical and nutritional cycling, and metabolite and vitamin biosynthesis groups. Besides, in *C. reniformis* symbiotic communities probiotic microbes orchestrating inter-taxa interactions were of particular relevance (Supplementary Table 3). Autotrophic CO₂ fixing microbes were introduced in vent core microbiomes of both host species, including cyanobacteria, which fix CO₂ via the Calvin–Benson–Bascham cycle (Durall and Lindblad, 2015; Klawonn et al., 2016), *Nitrospira*, which perform the reductive tricarboxylic acid cycle and the chemoautotrophic Gammaproteobacteria UBA10353 (Burgsdorf et al., 2022). Many vent-associated symbionts might be further involved in DOM cycling, such as DOM consuming *Endozoicomonas* and *Nitrospira*, as well as DOM degrading Dadabacteria. Instead, DOM consuming Poribacteria were associated with the control site. These trends may suggest that inorganic carbon fixation as well as DOM consumption and transformation could be involved in acclimation processes of sponge holobionts towards OA. Nitrogen cycling within sponge hosts is accomplished by microbial partners (Bayer et al., 2008; Fiore et al., 2015; Webster and Thomas, 2016; Pita et al., 2018) and many typical sponge symbionts are enriched in nitrogen metabolism genes (Fan et al.,

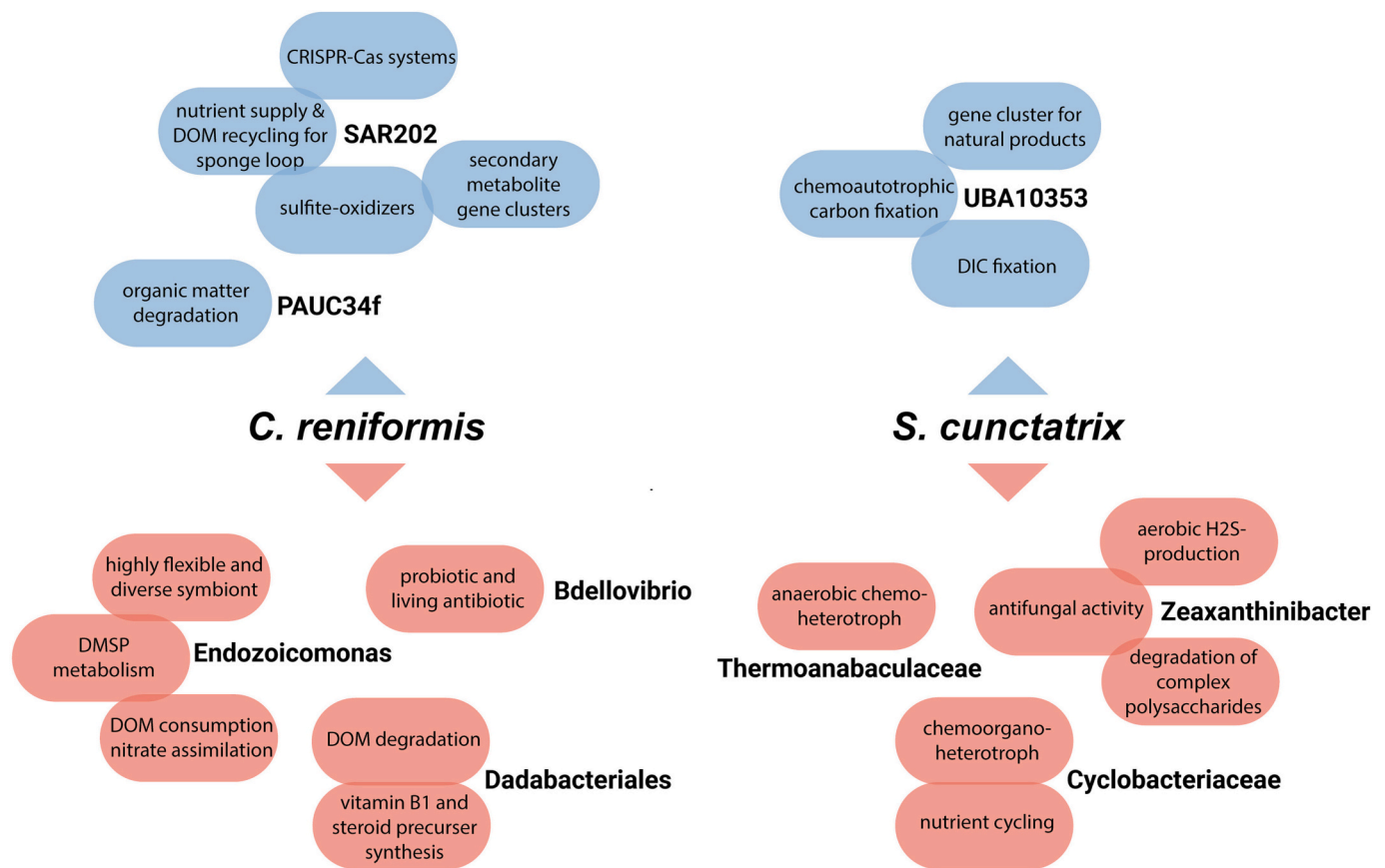


Fig. 6. Differentially abundant core microbes and their putative functions. Overview of differentially abundant core microbes for *C. reniformis* and *S. cunctatrix* sponges (Songbird and/or DEICODE analyses). Red boxes refer to putative functions upregulated under vent conditions (lower pH), while blue boxes present putative functions upregulated under “control” conditions (“normal” pH). Putative assigned functions are based on literature (Supplementary Table 8).

Box 1

Ecological importance of differential core taxa in mediating holobiont responses to OA

1. Consistency in presence: Core symbionts are consistently present in the symbiotic association with the sponge host across different environmental conditions, indicating a stable and essential relationship.
2. Functional relevance: Core symbionts are able to safeguard essential services, such as nutrient and biochemical cycling, (primary and secondary) metabolite and vitamin production, or inter-taxa symbiosis orchestration and protection against pathogens under a range of environmental scenarios.
3. Ecological versatility: Core symbionts that can flourish in vent and control conditions, possess ecological versatility, and can be suitable candidates to assist in host responses to OA.
4. Compositional key regulators: Core symbionts that change in differential abundance across diverse pH conditions may modulate microbiome composition and function, promoting acclimatization to OA.
5. Biomarker potential: Identifying differentially abundant core symbionts in vent and control conditions can afford diagnosis parameters about the health status of sponge microbiomes.

2012; Bayer et al., 2014; Li et al., 2016). In our vent samples increased abundances were reported for the nitrate-reducing *Candidatus Kaiserbacteria*, the nitrate-assimilating *Endozoicomonas*, and two ammonia-oxidizing symbionts, AqS1 (Nitrosococcaceae) and *Nitrospira* (Mohamed et al., 2010; Danczak et al., 2017; Neave et al., 2017; Taylor and Kurtz, 2020). Sulfur-oxidizing bacteria were also influential in our vent sponges; *S. cunctatrix* showed increased associations with sulfide-oxidizing Gammaproteobacteria, including Thioglobaceae, UBA10353 and AqS1 (Baltar et al., 2022), but also *C. reniformis* with SAR202 (Mehrshad et al., 2018). Many bacterial phyla associated to both sponge hosts seem to possess pathways to synthesize thiamine (vitamin B1), including Acidobacteriota, Actinobacteriota, Bacteroidota, Chloroflexi, Dadabacteria, Gemmatimonadota, Latescibacterota, Nitrospinota, Nitrospirota, Poribacteria and Proteobacteria (Engelberts et al., 2020). In *C. reniformis* B-vitamin groups appear to be compensated, with Poribacteria being replaced by Dadabacteriales (Thomas et al., 2010) in vent sponges, suggesting an extensive metabolic redundancy to ensure B-vitamin production under low pH. *Bdellovibrio* are considered probiotic biocontrol agents that prey on gram-negative opportunists and pathogens ('living antibiotics'), and can modulate microbiome relationships (Cavallo et al., 2021). In *C. reniformis* *Bdellovibrio* seem to be core regulators (Mazzella et al. unpublished) for inter-taxa dynamics, promoting flexible microbiome rearrangements under changing conditions, including OA. Thermoanabaculaceae, constituting anaerobic chemoautotrophs from volcanic environments, were associated to vent sponges, and in particular to the core of *S. cunctatrix*, likely performing as low pH adapted symbionts (Dedysh et al., 2020). Finally, core zeaxanthin pigment-producing *Zeaxanthinibacter* in vent *S. cunctatrix* may represent rather opportunist/infective associates, able to degrade complex polysaccharides from host structures (Sang and Kim, 2012). Besides some cases, it can be recognized a broad overlapping in positive fitting functions throughout different sponge-associated microbes. This indicates a key role among symbiont communities in providing functional redundancy and host stability, in the face of changing environments. Although many functions remain speculative, they offer starting points for future investigations.

4. Conclusions

In the last decade, research utilizing natural CO₂ vents as natural analogues for ocean acidification conditions has provided new insights into the long-term impacts on species, communities, and ecosystems (González-Delgado and Hernández, 2018; Aiuppa et al., 2021). The significance of conducting studies in these natural environments lies in the fact that the spatial carbonate chemistry gradient mimics the temporal continuum of future CO₂ levels. While these investigations have been crucial in advancing our understanding of OA effects, they also offer valuable opportunities to evaluate the cumulative impacts of

multiple climatic drivers on forthcoming global ocean ecosystems. By integrating carbonate chemistry conditions with diverse warming scenarios and extreme climatic events, such as marine heatwaves (especially during and after the peak of summer temperatures), or by manipulating temperature in situ, we could gain new insights into the anticipated conditions of the future global ocean and actively contribute in predicting how species will respond.

This study shed light on the microbial dynamics of two Mediterranean sponge species in response to natural OA. Results suggest that both, *C. reniformis* and *S. cunctatrix* restructured their microbiome via changes in microbial relative abundances and differentially abundant taxa (symbiont shuffling), as well as through the acquisition or depletion of new ASVs in the vent site (symbiont switching), in either case reshaping the core composition.

4.1. *Chondrosia reniformis*

Chondrosia reniformis displayed no morphological impairment and was highly abundant at the CO₂ vent site, suggesting that *C. reniformis* holobionts cope well with low pH conditions. The microbial community displayed changes in beta diversity metrics, including the acquisition and depletion of ASVs (symbiont switching), and changes in the relative abundances of microbial taxa (symbiont shuffling). Although the microbial community changed across sites, revealing a reduction in the number of core taxa and alpha diversity in vent specimens, mostly microbes with putatively similar functions were maintained. This may imply broad microbial-metabolite functional redundancy for species resilience. Several taxa, including *Endozoicomonas*, Dadabacteriales, *Bdellovibrio*, and *Candidatus Kaiserbacteria*, were differentially more abundant in vent specimens, yet within-community variabilities were low and overall classified as normobiotic. All this suggests that *C. reniformis* reshaped its microbiome as a positive adaptation, rather than as a bypass stress arrangement, with the possibility to develop, over generations, a microbially acclimatized symbiotic phenotype at the vent populations.

4.2. *Spirastrella cunctatrix*

Spirastrella cunctatrix assemblages were apparently abundant, but differed conspicuously across control to vent sites. Vent specimens showed morphological signs of scrawny growth, through a remarkable lower body surface area, smaller water canals and oscula, paler coloration and general signs of tissue thinning. Microbiome dynamics further suggested signs of microbial instability and incipient dysbiosis, according to dysbiosis scores, and by reporting increases in alpha diversity (likely including opportunistic strains), and a shift from sponge enriched microbes towards seawater microbes. The number of core taxa notably increased in vent hosts. Differentially abundant core members included

associates adapted to OA, and likely contributing in nutrient cycling (Cyclobacteriaceae and Thermoanabaculaceae), but also seeming opportunistic microbes (*Zeaxanthinibacter*). Such profound core reshaping and intragroup microbiome variability could pinpoint arbitrary stress rearrangements. Therefore, vent *S. cunctatrix* seem to be thriving under sub-optimal fitness, without developing reliable acclimatized populations.

The variety of responses described in microbial, physiological, morphological, and biochemical patterns indicate that not all sponge species can be classified as winner or loser taxa in future climate change scenarios (Lesser and Slattery, 2020; this study), as previously generalized (Bell et al., 2013, 2015, 2018a, 2018b). Indeed, host species-specificity remains decisive in holobiont acclimatization (e.g., Morrow et al., 2015; Glasl et al., 2018; Kandler et al., 2018; this study). Microbiome rearrangements in the form of positive adaptations appear to be beneficial for sponge hosts in a fluctuant environment, provided that metabolic homeostasis is maintained (Botté et al., 2019; Efremova et al. unpublished). Overall, symbiosis continues to demonstrate itself as the key evolutionary engine in species adaptation, and resilience for both present and future oceans.

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CRedit authorship contribution statement

Jana Efremova: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. **Valerio Mazzella:** Methodology, Software, Supervision, Visualization, Writing – review & editing. **Alice Mirasole:** Data curation, Methodology, Validation, Visualization, Writing – review & editing. **Nuria Teixidó:** Data curation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Laura Núñez-Pons:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Data availability

The datasets generated for this study can be found in the NCBI database <https://www.ncbi.nlm.nih.gov/sra/PRJNA926125>.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2024.170171>.

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