



Effects of Seasonal Anoxia on the Microbial Community Structure in Demosponges in a Marine Lake in Lough Hyne, Ireland

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ABSTRACT Climate change is expanding marine oxygen minimum zones (OMZs), while anthropogenic nutrient input depletes oxygen concentrations locally. The effects of deoxygenation on animals are generally detrimental; however, some sponges (Porifera) exhibit hypoxic and anoxic tolerance through currently unknown mechanisms. Sponges harbor highly specific microbiomes, which can include microbes with anaerobic capabilities. Sponge-microbe symbioses must also have persisted through multiple anoxic/hypoxic periods throughout Earth's history. Since sponges lack key components of the hypoxia-inducible factor (HIF) pathway responsible for hypoxic responses in other animals, it was hypothesized that sponge tolerance to deoxygenation may be facilitated by its microbiome. To test this hypothesis, we determined the microbial composition of sponge species tolerating seasonal anoxia and hypoxia *in situ* in a semienclosed marine lake, using 16S rRNA amplicon sequencing. We discovered a high degree of cryptic diversity among sponge species tolerating seasonal deoxygenation, including at least nine encrusting species of the orders Axinellida and Poecilosclerida. Despite significant changes in microbial community structure in the water, sponge microbiomes were species specific and remarkably stable under varied oxygen conditions, which was further explored for *Eurypon* spp. 2 and *Hymeraphia stellifera*. However, some symbiont sharing occurred under anoxia. At least three symbiont combinations, all including large populations of *Thaumarchaeota*, corresponded with deoxygenation tolerance, and some combinations were shared between some distantly related hosts. We propose hypothetical host-symbiont interactions following deoxygenation that could confer deoxygenation tolerance.

IMPORTANCE The oceans have an uncertain future due to anthropogenic stressors and an uncertain past that is becoming clearer with advances in biogeochemistry. Both past and future oceans were, or will be, deoxygenated in comparison to present conditions. Studying how sponges and their associated microbes tolerate deoxygenation provides insights into future marine ecosystems. Moreover, sponges form the earliest branch of the animal evolutionary tree, and they likely resemble some of the first animals. We determined the effects of variable environmental oxygen concentrations on the microbial communities of several demosponge species during seasonal anoxia in the field. Our results indicate that anoxic tolerance in some sponges may depend on their symbionts, but anoxic tolerance was not universal in sponges. Therefore, some sponge species could likely outcompete benthic organisms like corals in future, reduced-oxygen ecosystems. Our results support the molecular evidence that sponges and other animals have a Neoproterozoic origin and that animal evolution was not limited by low-oxygen conditions.

KEYWORDS Demospongiae, Porifera, *Thaumarchaeota*, anoxia, deoxygenation, host-microbe interactions, microbiome

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Ocean anoxia and hypoxia have become major stressors for many marine organisms. Indeed, oxygen minimum zones (OMZs) and coastal hypoxic areas will most likely expand in the future (1–5), leading to habitat and biodiversity losses (5–8), where oxygen depletion is caused by both natural and anthropogenic influences (9, 10). While motile species can escape such hypoxic/anoxic areas, many sessile organisms like sponges (Porifera) and corals (Cnidaria) must either cope with these extremes or suffer mass mortalities, as observed in tropical dead zones (11). Nevertheless, the lethal thresholds of, and potential adaptations to, deoxygenation are understudied (1–5) in these organisms (12), particularly in sponges.

Sponges are common, cosmopolitan filter feeders that pump water through their bodies to filter and ingest nutrients and microorganisms (13, 14). Previous *ex situ* experiments have shown that sponges, including *Geodia barretti* (15), *Tethya wilhelma* (16), *Halichondria panicea* (17), *Haliclona pigmentifera* (18), and *Vazella pourtalesii* (19), have a high tolerance of hypoxia. For instance, *T. wilhelma* can maintain normal transcription at $\sim 0.5 \mu\text{M O}_2$ (16), despite lacking key components of the hypoxia-inducible factor (HIF) pathway, which regulates hypoxic responses in other invertebrates (16). However, *T. wilhelma*, *H. panicea*, and *H. pigmentifera* became stressed, sometimes fatally, during anoxia (16–18). Some marine sponge species also tolerate hypoxia and even anoxia in their natural environment (20–24), and gemmules from freshwater sponges can survive months of anoxia (25). Nevertheless, hypoxia-induced mortality has been reported *in situ* for the demosponges *Aaptos simplex* and *Homaxinella amphispicula* (21), so responses are likely species specific. The current global distribution and depth range of sponges (26) overlap with those of hypoxic areas worldwide (2), indicating that many sponges may even thrive in hypoxic environments, perhaps due to limited competition. Therefore, some sponges have likely developed alternative adaptation strategies to tolerate variable and low-oxygen conditions independent of the HIF pathway.

Sponges can harbor stable and sometimes diverse microbial communities that can constitute up to 50% of their biomass (27, 28); together, the host and microbiome are referred to as the sponge “holobiont” (29). Sponge holobionts play a key role in marine ecosystems, contributing to reef formation, benthic-pelagic nutrient coupling, and biogeochemical cycling (30–32). Some of these sponge-microbe associations are highly stable under environmental stressors, including elevated temperature (33–35), eutrophication, and sedimentation (36), and some symbionts are sponge specific, meaning that they occur negligibly in the environment (37). Stable sponge-microbe associations have also been found across large geographic distances (38). However, microbial communities across sponge species vary substantially in diversity, structure, and abundance (39), and there can be selection for divergent microbiomes, even among related sponge lineages (40).

Within a specific holobiont, symbiotic microbes may increase sponge fitness by providing food (through carbon fixation or direct ingestion of symbionts), recycling of nutrients and waste products, and/or the production of secondary metabolites for predator defense or other functions (30, 31). Many sponge species form symbioses with *Nitrosopumilus*-like ammonia-oxidizing *Archaea* (AOA) and/or *Nitrospira* spp. nitrite-oxidizing *Bacteria* (NOB) (19, 40–44). Each of these prokaryotic groups utilizes sponge waste ammonia for nitrification. Similar microbes are active in hypoxic waters and contribute to biogeochemical cycling processes (45). For example, *Nitrospira* spp. accounted for 9% of the total microbial community in the OMZ of the Benguela upwelling system (46). Also, *Nitrosopumilaceae*, a widespread and dominant AOA in many OMZs, plays a significant role in ammonium oxidation therein (47, 48). Thus, many of these common sponge symbionts might be adapted to hypoxia, and it has been suggested that the presence of AOA symbionts, and other facultative anaerobes, within a glass sponge signified holobiont adaptation to mild, persistent environmental hypoxia (19). Furthermore, some symbionts have anaerobic metabolisms, including sulfate reducers (49), denitrifiers, and anaerobic ammonium oxidizers (anammox

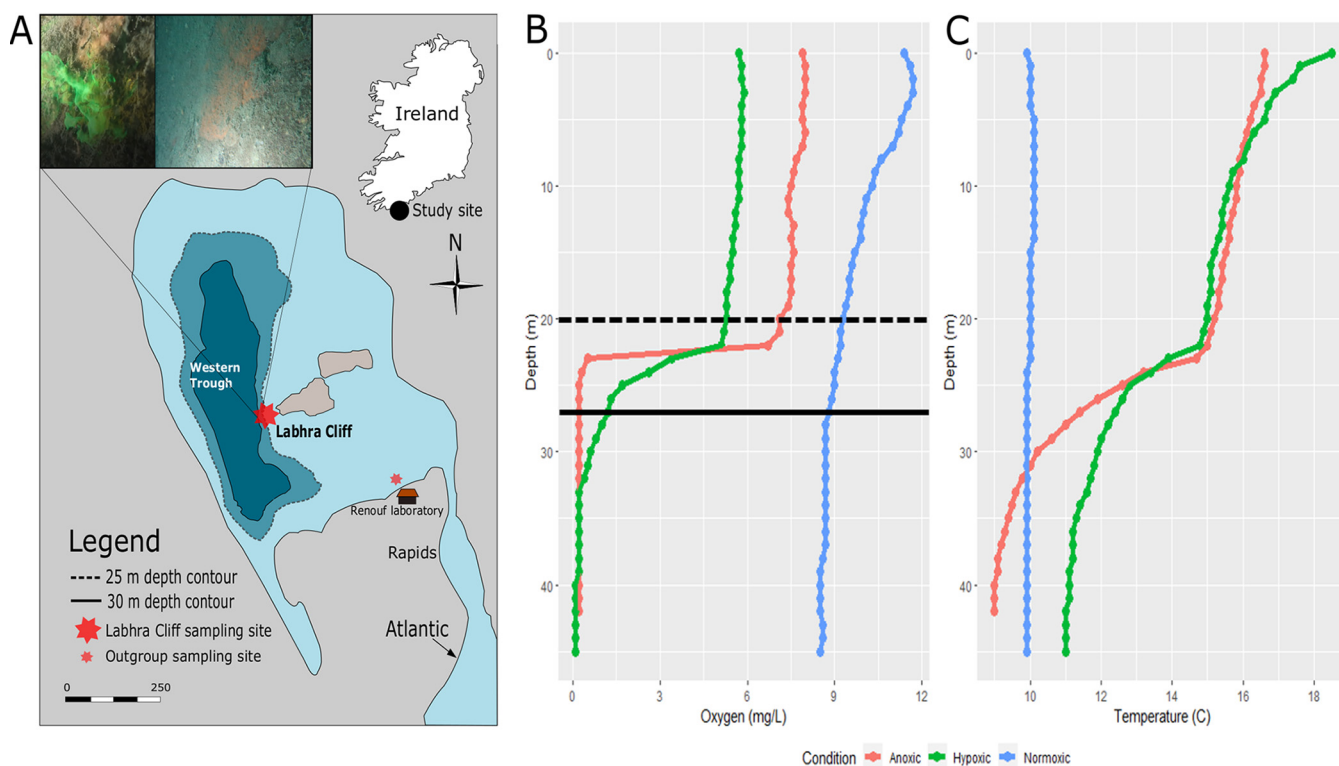


FIG 1 Map of Lough Hyne with sampling sites and oxygen and temperature profiles taken from the middle of the Western Trough in Lough Hyne. (A) Map of Lough Hyne showing sampling sites and *in situ* pictures of encrusting sponges at 27-m depth. Upper left image, the sponge actively pumping as visualized using fluorescein dye under hypoxic conditions; upper right image, a sponge without dye. (B) Dissolved oxygen concentrations versus depth for the three different sampling trips, corresponding to three different oxygen conditions at ~27 m (solid line): July 2018, anoxic (red), July 2019, hypoxic (green), and April 2019, normoxic (blue). Samples were collected at ~27 m (solid black line, below the thermocline) and at ~20 m (dashed black line, above the thermocline) for comparison across time and oxygen condition. (C) Temperature versus depth during the different sampling oxygen conditions.

bacteria) (50). Localized and widespread anoxia is common within sponge tissue, even within oxygenated environments, due to pumping cessation, and this allows for anaerobic metabolism of the symbionts (49). Such anaerobic metabolism may be crucial for nutrient cycling.

The low-oxygen tolerance of the sponge holobiont was probably crucial throughout evolutionary history. The symbiosis of sponges with microbes dates back hundreds of millions of years (51–53), indicating adaptations of holobionts to environmental changes and strong coevolution throughout Earth's history, including long periods of anoxia/hypoxia (54–58). For example, hypercalcified sponges likely survived the late and end-Permian mass extinction and deoxygenation because of their oxygenic cyanobacterial symbionts (59). Based on morphology (60) and phylogeny (61), we assume that the last common ancestor of metazoans was sponge-like. Therefore, the low-oxygen tolerance in early metazoans, similar to that of modern sponges, could have permitted their evolution under lower oxygen concentrations (1 to 20% of present levels [62]) in the Neoproterozoic Era (16, 63, 64). Although the symbiont status of early metazoans is unknown, sponges evolved in a world rich in microbes (65), and hypoxic tolerance of modern sponge holobionts could date back to these associations.

Based on the microaerophilic and anaerobic metabolisms of some sponge symbionts (66, 67), and the evolutionary importance of hypoxic tolerance, we hypothesized that specific microbes or microbial compositions help sponges survive in low-oxygen or even anoxic environments. To test this hypothesis, sponge, sediment, and water samples from a semienclosed marine lake (Lough Hyne, Ireland) (Fig. 1A) were sampled and analyzed using 16S amplicon sequencing to determine microbial community structure and molecular barcoding to identify sponge species. Lough Hyne is characterized by its unique ecological, physical, and chemical conditions (see reference 68).

This lough was a freshwater basin until around 4,000 years ago (69) but became a marine basin due to rising sea levels and intrusion from the Atlantic Ocean over a shallow sill. Water retention in the lough is between 14 and 41 days (70, 71). In the summer, a seasonal anoxia/hypoxia layer forms at a depth of approximately 25 m. The water beneath this layer becomes anoxic and enriched in H_2S , NH_4^+ , and Mn^{2+} (68, 69). Lough Hyne also harbors a remarkably high biodiversity of sponges, and some of these species have been reported to survive seasonal anoxia (72), making it a natural laboratory to study the effects of variable oxygen on sponge-microbiome associations.

Samples were taken above and below the thermocline, and during different seasons, to allow a comparison of a wide range of oxygen conditions, from normoxia to anoxia. In addition, three sponge species were sampled outside the seasonally anoxic site for comparison (Fig. 1A). This study aimed to answer the following questions. (i) What sponge species survive seasonal anoxia/hypoxia in Lough Hyne? (ii) Is there a symbiont composition that corresponds to deoxygenation tolerance? And, finally, (iii) what implications does sponge holobiont deoxygenation tolerance have for early animal evolution in low-oxygen environments and in future oceans? To our knowledge, this is the first study to comparatively investigate the microbial community composition of sponges under different *in situ* oxygen levels.

RESULTS

Physical data. Sampling was performed in July 2018, April 2019, and July 2019, when oxygen conditions at Labhra Cliff (Fig. 1A), between 25 and 30 m deep, were anoxic, normoxic, and hypoxic, respectively (Fig. 1B). The hypoxic conditions in July 2019 were anomalous compared to the normally anoxic summer conditions below 25 m at this site and likely occurred due to heavy storm and wind mixing of waters in the lough. These storm events increased mixing across the thermocline and pushed resultant anoxia to a depth of at least 33 m, which is below the depth of the cliff/dive site (Fig. 1B and C). Measurements taken by conductivity, temperature, and depth (CTD) in the center of the Western Trough (4 casts to 40 m) and at Labhra Cliff (2 casts to ~30 m) indicated that total anoxia occurred at 40 m depth (see Fig. S1B in the supplemental material), while conditions between 25 and 30 m were still “hypoxic” (50 to 150 μM) (Fig. S1A). Although CTD measurements were not performed in July 2018, the total depletion of oxygen under the “anoxic” condition was verified by the presence of sulfide in the water, which cannot persist in the presence of oxygen. Sulfide concentrations in two samples were 0.90 μM and 0.19 μM . While these samples were quickly frozen after collection, sulfide was not stabilized with ZnS acetate until they were returned to the lab, so the actual sulfide concentrations were likely higher. Furthermore, following anoxic collections, all water samples and equipment including scuba gear had the characteristic smell of sulfide. The presence of photosynthetic pigments (Fig. S2) indicated that there was a potential food source for sponges regardless of oxygen condition.

Diversity of sponges surviving seasonal anoxia/hypoxia. Molecular barcoding of two independent marker genes, the mitochondrial *cox1* and the ribosomal partial 28S C region, revealed 30 demosponge specimens from the Labhra Cliff site. The majority of specimens belonged to the family Raspailiidae (subfamily Raspailiinae) of the order Axinellida, except for a *Mycale* sp., a noncarnivorous poecilosclerid sponge collected under anoxic conditions (Fig. 2; Fig. S3), but all were visually similar (orange-red, encrusting) *in situ*. Within the Axinellida, species of the genera *Eurypon*, *Endectyon*, *Raspaciona*, and *Hymenaphia* (Fig. 2) were sampled. This includes seven species that are definitely exposed to seasonal anoxia/hypoxia, namely, *Eurypon* spp. 2 ($n=14$), a *Eurypon clavigerum* ($n=2$), a *Eurypon* cf. *cinctum* ($n=1$), *Hymenaphia stellifera* ($n=8$), *Endectyon* spp. 1 ($n=2$), *Endectyon* spp. 2 ($n=2$), and a *Raspaciona* sp. ($n=1$) (Fig. 2; Fig. S4). However, given the challenging sampling conditions at this site in the lough (see Materials and Methods), it is possible that other species, such as *Amphilectus* spp., *Rhizaxinella* spp., and *Hymeniacidon perlevis*, collected above 24 m, may also be present in the anoxic/hypoxic layers but were simply not sampled during our dives. Notably, all species sampled from below 24 m were encrusting in their growth form.

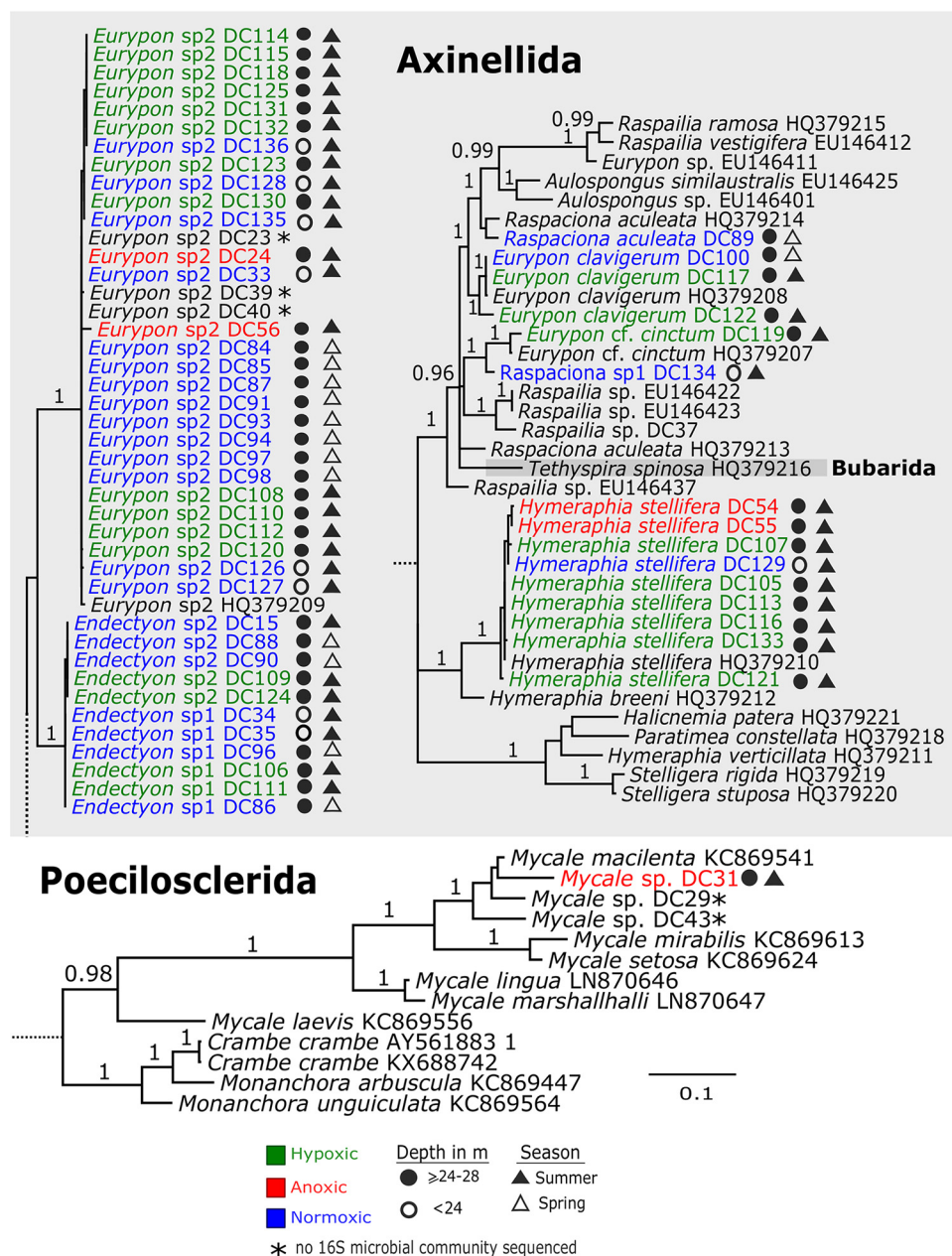


FIG 2 28S Bayesian inference (BI) phylogeny of sponges from Lough Hyne (indicated by DC numbers after taxon names). For visualization, subtrees were pruned from the complete phylogenetic tree (see Fig. S3 in the supplemental material). Posterior probability values of >0.95 are given above branches. See Fig. S4 for *cox1* BI phylogeny.

Systematically, the genera *Eurypon*, *Hymeraphia*, and *Endectyon* are polyphyletic (Fig. 2; Fig. S3). Both markers (28S C region and *cox1*) indicated that *Eurypon* spp. most likely represent a complex of species, which will be taxonomically resolved in the future using more informative markers and holotypes. All sponges exposed to fluorescein dye at 27 m were pumping under both normoxic and hypoxic conditions (Fig. 1A). Fluorescein dye was not introduced to sponges under anoxia due to logistical constraints.

Microbial communities. A total of 89 sponge, water, and sediment samples were collected from Labhra Cliff, in which 4,677 operational taxonomic units (OTUs) were identified. An additional 114 OTUs were present in the 14 outgroup samples,

TABLE 1 PERMANOVA results for the focused subset after 1,000 permutations^a

Factor	df	F	R ²	P
Oxygen	2	10.2	0.0777	<0.001
Sample type	3	59.6	0.678	<0.001
Oxygen × sample type	5	4.03	0.0765	<0.001
Residuals	44			

^aF = F statistic; R² = R squared.

consisting of sponges sampled in normoxic waters away from Labhra Cliff (Fig. 1). For all samples, the average Shannon index was 3.1 and approximately 500 OTUs were identified in each sample. The data were filtered into a “focused subset” that included sponge species with replication across all oxygen conditions. The focused subset included the sponges *Eurypon* sp. 2 and *Hymenophyllum stellifera* as well as water and sediment samples, but notably, no sediment samples under anoxic conditions were collected.

In total, the focused subset mentioned above comprised 55 samples, containing 4,518 different OTUs (~600 per sample on average) with an average Shannon index of ~3 per sample. A permutational multivariate analysis of variance (PERMANOVA) performed on the focused subset indicated that although there were some microbial community shifts based on oxygen condition and the interaction between oxygen condition and sample type, these factors accounted for only 7.33 and 7.84% of the variance in OTUs within the data set, respectively (Table 1). Most of the variance (67.8%) (Table 1) in microbial community structure came from the type of sample, i.e., *Eurypon* sp. 2, *H. stellifera*, water, or sediment, which was also clear from a principal-component analysis (PCA) (Fig. 3A).

In the PCA, samples grouped definitively by sample type, but the different oxygen conditions did not form clear subgroups even within each sample type, except for the community structures in the water samples (Fig. 3A). When the ordination was constrained by oxygen condition in a canonical correspondence analysis (CCA), anoxic samples appeared to separate out more clearly, but this separation was driven by the communities present in the water samples (Fig. 3B, plus symbols). The OTUs driving this separation were identified, extracted, and plotted in a heat map (Fig. 3C), further demonstrating that changes in relative abundance within the water microbial community were not reflected in the microbiomes of either sponge species or within the sediment. Even if water samples were removed from the microbial community analysis, the OTUs driving the separation by oxygen condition were still much more abundant in the water than in sponge samples and thus likely represent low levels of contamination from the water around the sponge and in the sponge water canals rather than stable symbioses. Outside the focused subset, the same pattern of OTUs in the water samples driving separation of microbial communities across oxygen conditions was observed for all Labhra Cliff samples (Fig. S5).

The relative abundances of the top seven most abundant OTUs were compared individually across sample type and oxygen condition within the focused subset (Fig. 4; Table S1). The less abundant OTUs (i.e., not top 7) all contributed less than 8% of the relative abundance of OTUs to any one species and did not exhibit notable changes in relative abundance with oxygen condition. In *Eurypon* sp. 2, the relative abundance of OTU1, a *Nitrosopumilus*-like *Thaumarchaeota* (Fig. 4), was approximately 40% across all oxygen conditions. From all other sample types, OTU1 was absent (relative abundance, 0%), except for *H. stellifera*, where one of the two individuals of *H. stellifera* appreciably took up OTU1 (relative abundance, 7.64%) under anoxia but the other did not (relative abundance, 0.01%). No significant differences were found between relative abundances of OTU1 in *Eurypon* sp. 2 under different oxygen conditions ($P > 0.05$) (Table S1).

Similar to that with OTU1, relative abundances of OTU2, a gammaproteobacterium (see Fig. 6 phylogeny), in *Eurypon* sp. 2 were approximately 20% throughout all oxygen

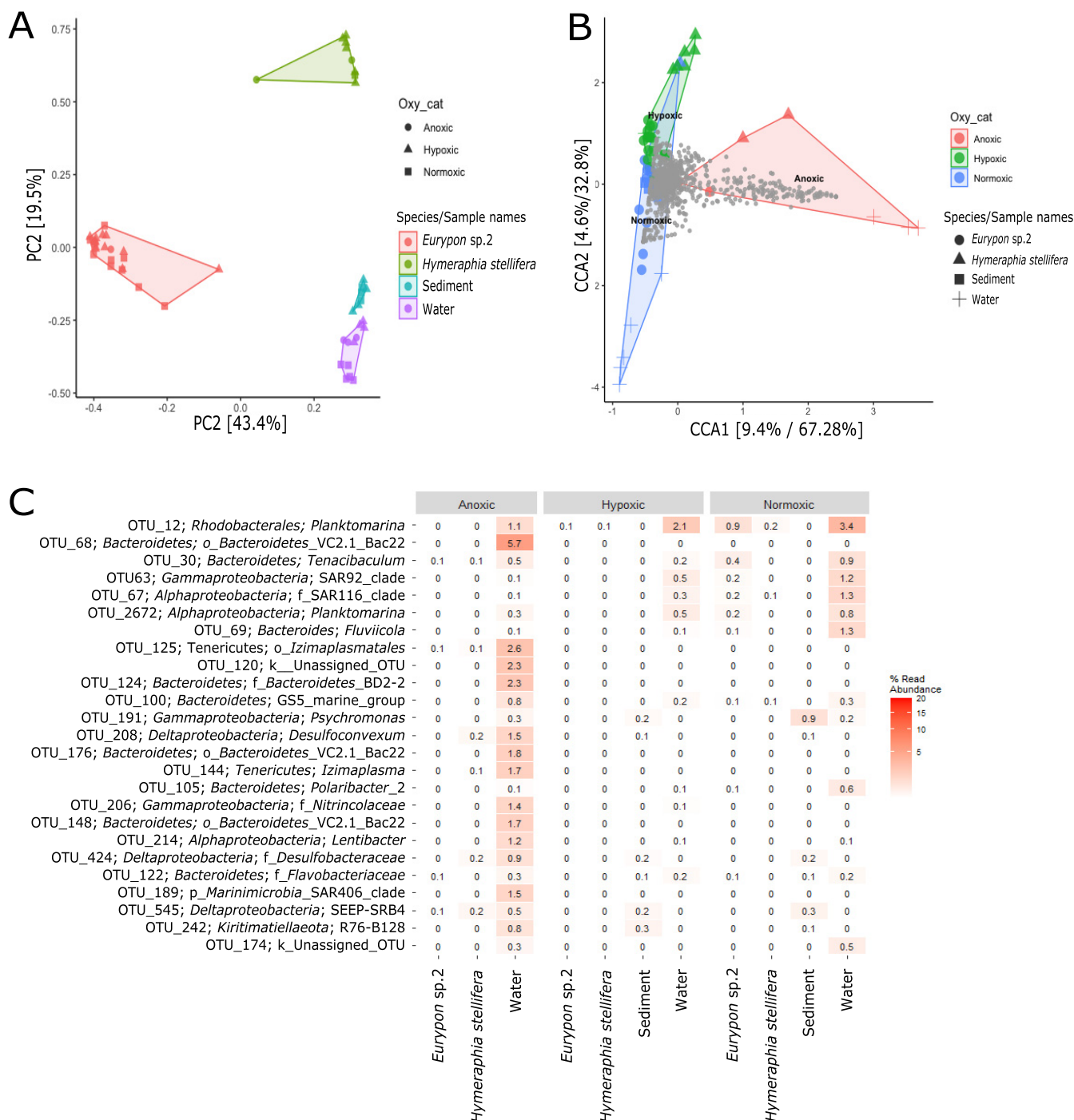


FIG 3 (A) PCA of the focused subset. (B) CCA of the focused subset. Individual OTUs are identified in gray. (C) Heat map of the OTUs (order level) driving the separation based on anoxia in the CCA, i.e., OTUs contained within the red shaded area in panel B.

conditions, and OTU2 was absent from all other sample types except for *H. stellifera* during anoxia. Within anoxic *H. stellifera*, relative abundances of OTU2 ranged from 5.75 to 0.01% for the two individuals. A Kruskal-Wallis rank sum of only samples from *Eurypon* sp. 2 revealed no significant differences in relative abundance of OTU2 between oxygen conditions ($P > 0.05$) (Table S1). In contrast, OTU3 and OTU7, a *Nitrosopumilus* sp. and a gammaproteobacterium (Fig. 5 and 6, see phylogenies), respectively, were more abundant in *H. stellifera* than in *Eurypon* sp. 2.

Unlike all other top OTUs (Fig. 4), OTU3 was also found within the sediment and

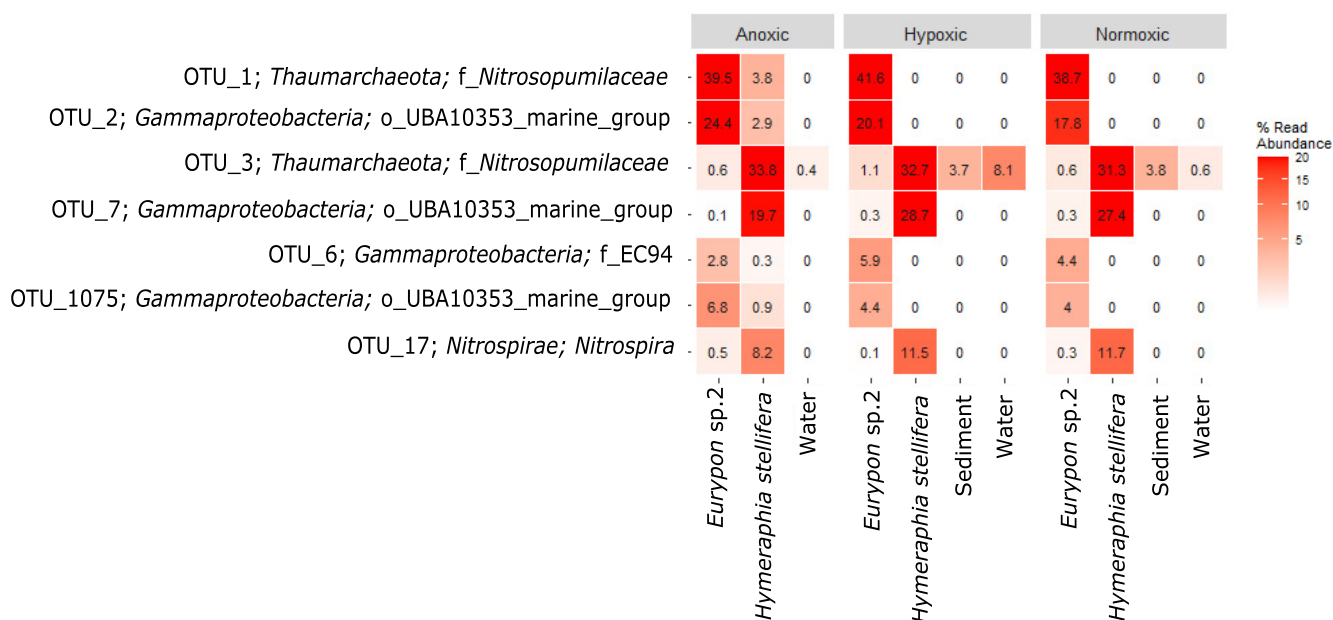


FIG 4 Heat map of top seven most abundant OTUs in the focused subset.

water in addition to sponge samples. Relative abundances in OTU3 were significantly higher in *H. stellifera* than in any other sample type ($P < 0.001$); however, there were no significant differences between the relative abundances of OTU3 within *H. stellifera* under different oxygen conditions ($P > 0.05$) (Table S1). Relative abundances of OTU3 within *H. stellifera* were stable at approximately 32% under all oxygen conditions. Relative abundances of OTU3 were significantly lower in *Eurypon* sp. 2 than in any other sample type, 0.6 to 1.1% ($P < 0.05$), but no significant changes were observed between oxygen conditions. Within the water and sediment, average relative abundances of OTU3 ranged from 0.4 to 8.1% but were not significantly different from one another. The relative abundance of OTU3 was significantly higher in hypoxic waters than normoxic waters ($P < 0.05$) (Table S1), but no other significant differences were detected.

Overall, relative abundances in OTU7 were significantly greater in *H. stellifera* than in any other sample type ($P < 0.001$), and these differences held during pairwise comparisons ($P < 0.001$) (Table S1). There was a significant decrease in the relative abundance of OTU7 in *H. stellifera* under anoxia compared to that under hypoxia and normoxia ($P < 0.001$; mean relative abundances of 19.7, 28.7 and 27.4%, respectively) (Fig. 4). Relative abundances of OTU7 also appeared to decrease within *Eurypon* sp. 2 from 0.3 and 0.4% under normoxia and hypoxia to 0.1% under anoxia, but this difference was not significant ($P > 0.05$).

The *Gammaproteobacteria* OTU6 and OTU1075 were absent in the sediment and water samples but present in all *Eurypon* spp. 2 samples and present only under anoxia in *H. stellifera*. For *H. stellifera* under anoxia, the relative abundances of OTU6 were 0.549 and 0.002% for the two replicates, while the abundances of OTU1075 were 1.89 and 0% for the two replicates. No significant changes in the relative abundance of OTU6 were observed in *Eurypon* sp. 2 across oxygen conditions. The relative abundances of OTU1075 increased significantly ($P < 0.05$) from approximately 4% under hypoxia and normoxia to approximately 6% under anoxia. The *Nitrospira* OTU17 was also present in both sponge species under all conditions, but relative abundances were significantly higher in *H. stellifera* (~8 to 12%) than in *Eurypon* sp. 2 (~0.1 to 0.5%). No significant changes in relative abundance in OTU17 were observed based on oxygen condition.

Considering the full data set (Fig. 7), all sponge species collected from Labhra Cliff



FIG 5 Archaeal maximum likelihood phylogeny of partial 16S rRNA gene (292 bp), with bootstrap support values (1,000 replicates; GTRCAT model) given for nodes with 70% or greater. OTUs of interest are marked in red.

contained only a few key OTUs that constituted between 30 to 65% of their microbiomes, depending on the sponge species, and these patterns were generally host species specific or genus specific (Fig. 7). This trend was also true when relative abundance was examined at the microbial class level (Fig. S6). These key OTUs (OTU1 to 3, OTU4, OTU5, and OTU7) were absent in outgroup samples with the exception of OTU3,



FIG 6 Bacterial maximum likelihood phylogeny of partial 16S rRNA gene (292bp), with bootstrap support values (1,000 replicates; GTRGAMMA model) reported for nodes with 75% or greater. Sponge OTUs including all *Delta*- and *Gammaproteobacteria* OTUs from heat maps in Fig. 3C, 4, and 7 from this study are highlighted in red. The OTUs that were absent or negligible in sponge samples and present only in anoxic water are marked with an asterisk.

under anoxia that requires further study (see “Potential adaptations of the sponge host to seasonal anoxia”). Regardless of the role of the microbiome in deoxygenation tolerance, our observations of Lough Hyne sponges have important implications for ancient and future marine environments.

Sponge diversity at a seasonally anoxic site. There was a high level of cryptic diversity/speciation within the orange-red encrusting sponges, and at least eight such species are confirmed here as tolerant to seasonal anoxia and hypoxia (*Eurypon clavigerum*, *Eurypon* cf. *cinctum*, *Endectyon* sp. 1, *Endectyon* sp. 2, *Eurypon* sp. 2, *H. stellifera*, a *Mycale* sp., and a *Raspaciona* sp.). In addition to some *Eurypon* species and *H. stellifera*, Bell and Barnes (20) also noted the presence of a whitish encrusting species, *Paratimea constellata*, and an arborescent species, *Stelligera rigida* (now *Stelligera montagui* [73]), both Axinellida, at 30 m on Labhra Cliff. In the present study, *P. constellata* was observed (but not sampled); however, *S. montagui* was not observed. Given that their relationships have not yet been fully resolved, we suspect that the *Eurypon* species previously observed at this site form a species complex and are widely distributed among the Raspailiidae. This cryptic diversity was visible only with the present barcoding data from this study. Although they were present elsewhere in the lough, *Mycale* species were not previously reported at 30 m on Labhra Cliff, and *Raspaciona* species were not noted in previous surveys (20, 144). Since two unidentified massive sponges were also observed (B. W. Strehlow, personal observation) and “other” unidentified species were observed by Bell and Barnes (20), the actual diversity of sponges at this depth is higher than reported here. Despite this diversity, there was still a marked decrease in the overall abundance and diversity of sponge species in the seasonally anoxic waters below 24 m (20). Indeed, the sponge community at Labhra Cliff at 30 m is the most unique (based on Bray-Curtis similarity analyses) among those of sites in and around the lough because the diversity and abundance of sponges are limited to relatively few species, even though most of these species, including *Eurypon* spp. and *H. stellifera*, are part of the 25 most common species found in Lough Hyne (20). This observation indicates that sponges below 24 m are specifically adapted to this environment and that these adaptations could be reflected in their microbiomes.

Potential adaptations of the microbiome to seasonal anoxia. In the Labhra Cliff sponges, microbiomes were largely stable within individual species under different oxygen conditions, despite substantial changes in microbial populations in the water column. This stability suggests possible adaptations to deoxygenation within the microbiome. Similar stabilities of sponge microbiomes have also been noted in other studies where sponges have been exposed to sediment loading (36), thermal stress (74), and food shortage (75). Sponge microbiomes are significantly disrupted only when sponges experience physiological stress such as bleaching (76, 77), necrosis (34, 78), disease (79), or mortality (36). Therefore, the stability in the microbiomes we observed in Labhra Cliff sponges might indicate that the holobionts were “healthy” and adapted to periods of anoxia.

Although microbiomes were mostly host species specific across the whole data set (Fig. 7), some symbiont strategies were shared across sponge taxa in anoxia-tolerant species. In the four sponge species *Eurypon* sp. 2, *H. stellifera*, *Mycale* sp., and *Raspaciona* sp. (Fig. 7), three symbiont combinations were identified. These combinations were characterized by their most abundant OTUs, as follows: combination i, OTU1 and OTU2; combination ii, OTU3 and OTU7; and combination iii, OTU3 and OTU10 (Fig. 4 and 7). Combination i was exhibited by *Eurypon* sp. 2 and *Mycale* sp., and *H. stellifera* and *Raspaciona* sp. had combinations ii and iii, respectively. All combinations included high abundances of *Thaumarchaeota* (either OTU1 or OTU3). Combinations i and ii are also dominated by large populations of the *Gammaproteobacteria* OTU2 and OTU7, respectively. In addition to the *Thaumarchaeota* OTU3, combination iii was characterized by high abundances of the unknown OTU10. A summary of the various combinations is shown in Fig. 8 along with aerobic and anaerobic metabolic pathways that might be present in the holobionts, which are discussed in detail below.

While *Gammaproteobacteria* and *Thaumarchaeota* are common sponge symbionts

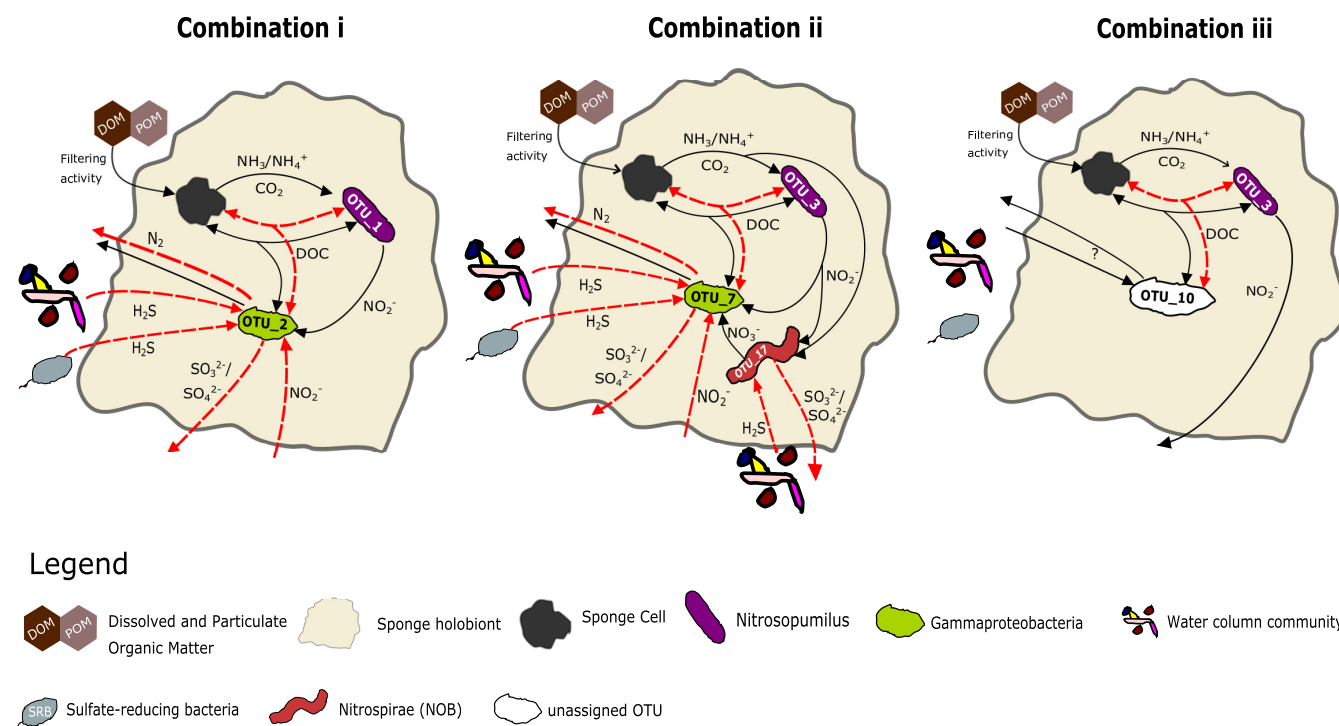


FIG 8 Hypothetical metabolic cycling processes in the sponge holobiont showing the three major symbiont combinations i, ii, and iii. Dashed, red arrows indicate the cycling processes under anoxia, and solid black arrows show processes under normoxic conditions.

and often cooccur in one host (40), the combinations of these specific OTUs at their high relative abundances may be unique to anoxia-tolerant species (Fig. 5 and 6). There was strong evidence of convergence toward combination i, since it was acquired only by *H. stellifera* under anoxia and was shared across a large host phylogenetic distance, i.e., between the pocilosclerid *Mycale* species and the axinellid *Eurypon* sp. 2. Although many emergent properties of sponge microbial communities, e.g., community complexity and interactions, are conserved across Porifera, it is rare that specific OTUs are shared across large host phylogenetic distances (40). This exceptional symbiont commonality as well as the acquisition of combination i by *H. stellifera* indicated that this combination may be better adapted to seasonal anoxia than combination ii. Combination iii, conversely, may represent a strategy just as successful as combination i, given its stability in anoxia within *Raspaciona* spp. (Fig. 7).

Both *Thaumarchaeota* OTUs were part of the *Nitrosopumilaceae* family, but OTU 1 is present only in sponges, making it sponge specific, whereas OTU 3 was also present in sediment and water samples, making it a generalist. The OTU 3 is part of a clade that contains more free-living *Thaumarchaeota* members, including *Nitrosopumilus maritimus* (Fig. 5), than symbionts. Conversely, OTU 1 forms a clade that is almost exclusively sponge or coral associated (Fig. 5). Based on the genomes of their close relatives, both OTU 1 and OTU 3 are likely AOA and could therefore oxidize sponge-derived ammonia, detoxifying the holobiont and potentially providing dissolved organic carbon (DOC) for the host that is ultimately sourced from chemolithotrophic carbon fixation (80, 81), making them integral parts of holobiont metabolism under normoxia (Fig. 8).

It has been reported that both ammonia oxidation rates and carbon fixation rates by an AOA symbiont are positively correlated within the sponge *Ianthella basta* (44). Similarly, *Thaumarchaeota* are the main drivers of nitrification in four cold-water sponges (41, 42). The AOA symbionts of a glass sponge living under mild hypoxia also possess elements of a facultatively anaerobic metabolism, including fermentation and fumarate, nitrite, and sulfite respiration (19). Although other AOA within the *Thaumarchaeota* do not generally include the aforementioned anaerobic elements, a terrestrial AOA

does have the capacity for aromatic amino acid fermentation (82), and it is possible that DOC transfer between symbiont and host continues via fermentation under anoxia (Fig. 8). The microbes themselves could also be a food source for the sponge (49, 83, 84).

Thus, it is possible that hypoxic environmental conditions are beneficial for the holobiont, given the low-oxygen requirements of sponges (16, 17) and the high abundance of *N. maritimus* in marine OMZs (85). Accordingly, the relative abundance of OTU3 significantly increased in the water (but not in any sponge species) during hypoxia but was not significantly different between anoxia and normoxia. Despite this increased abundance in the environment under hypoxia, populations of OTU3 were not significantly increased in *Eurypon* sp. 2 under the same conditions. Hypoxia, however, is not the “typical” condition between 25 and 30 m during the summer in Lough Hyne; instead, anoxia is typical in summer (68). Assuming that the sponges are active and pumping (see “Potential adaptations of the sponge host to seasonal anoxia”) and given that ammonium oxidation requires oxygen in *Archaea* (48), holobiont metabolisms may be very different under anoxia. Furthermore, *Thaumarchaeota* are functionally diverse (for examples, see reference 44), so the actual metabolisms and symbiotic functions of *Thaumarchaeota* in Lough Hyne sponges need to be verified under their respective oxygen conditions.

Like *Thaumarchaeota*, *Gammaproteobacteria* symbionts may contribute key functions to their holobionts, including some that provide tolerance to deoxygenation. Although no single *Gammaproteobacteria* OTU occurred in relative abundances greater than 5.2% in *Raspaciona* spp., symbiont combination i contained high relative abundances of the *Gammaproteobacteria* OTU2 and symbiont combination ii contained relatively high abundances of OTU7. Unlike the *Thaumarchaeota*, both OTU2 and OTU7 were sponge specific. Within the data set of focus taxa (Fig. 4), the significant decrease in OTU7 in *H. stellifera* during anoxia, compared to other oxygen conditions, may correspond to the appearance of OTU2, if both occupy the same niche. The same might be true of another gammaproteobacterium, OTU1075, which significantly increased in relative abundance in anoxia in *Eurypon* sp. 2 and was more closely related to OTU2 than OTU7 (Fig. 6).

The facultative anaerobe *Thioalkalispira microaerophila*, which can use sulfide as an electron donor and grows in micro-oxic conditions (86), as well as *Thiohalophilis thio-cyanatoydans*, which can grow anaerobically using thiosulfate as an electron donor and nitrite as an electron acceptor (86), are in the same clade as the sponge-specific *Gammaproteobacteria* (Fig. 6). Therefore, it is possible that the sponge-specific *Gammaproteobacteria* in our samples possessed both aerobic and anaerobic capacities and could remove exogenous, toxic sulfide from the holobiont under anoxia (Fig. 8). This latter process, however, would be dependent on an electron acceptor, probably nitrite, that could come from the environment under nitrogenous conditions or through unknown pathways within the holobiont under sulfidic conditions.

In addition to the two most abundant OTUs in combination ii, a *Nitrospira* (OTU17) was found in high relative abundances in *H. stellifera*. Although it was absent or significantly less abundant in other Labhra Cliff sponges (Fig. 7), OTU17 may perform important metabolic functions within the *H. stellifera* holobiont. The OTU17 is closely related to a *Nitrospira* (CcNi) that is associated with the sponge *Cymbastela concentrica*, and even though some *Nitrospira* spp. can completely oxidize ammonia to nitrate (commox), OTU17 may only oxidize ammonium to nitrite, as was predicted for CcNi (87). Although it was not significant, relative abundances of OTU17 decreased during anoxia in *H. stellifera*, which could be due to a lack of oxygen inhibiting the metabolism and growth of *Nitrospira* species.

Although *Nitrospira* spp. are also conspicuously absent (Fig. 3) (see also references 40 and 44) or inactive (42) in some sponge holobionts in general, CcNi symbionts in *C. concentrica* form close metabolic associations with the host and other microbes, including a member of the *Thaumarchaeota*, namely, CcThau (87). Coincidentally,

CcThau is more closely related to OTU3 than to OTU1 (Fig. 5), and therefore the co-occurrence of relatively large populations of OTU3 and OTU17 might indicate a coevolution between these two OTUs. The acquisition of OTU1 by *H. stellifera* could therefore disrupt these partnerships under anoxia (88), but this remains to be tested. It is also possible that OTU17, like some of its congeners, could perform comammox and/or hydrogen oxidation coupled to sulfur reduction under anaerobic conditions, making it well adapted for low-oxygen stress (89). In either case, the *H. stellifera* holobiont likely employed a separate metabolic strategy under normoxia than under anoxia and compared to hosts with symbiont combination i or iii.

The holobionts from the genus *Raspaciona* likely employ different metabolic strategies in response to anoxia. Like *H. stellifera*, they host large, stable populations of OTU3, but *Raspaciona* spp. do not acquire more OTU1 or any OTU2 populations under anoxia. Instead, *Raspaciona* spp. harbored large, stable populations of the unidentified OTU10 (Fig. 7), which was completely absent from all other samples except for one *Eurypon* cf. *cinctum*, taken under hypoxia. The unassigned OTU10 has an unknown metabolism; however, its stability in *Raspaciona* spp. through anoxia and normoxia, and its presence in *Eurypon* cf. *cinctum*, indicates that it may confer some degree of deoxygenation tolerance (Fig. 8).

Microbes associated with sulfate reduction and anammox were conspicuously absent or present in very low abundances in Labhra Cliff sponges. Probable sulfate-reducing OTUs were present in the anoxic water at much higher abundances than in any sponge species under the same conditions, but they were absent in all sponges under normoxic and hypoxic conditions (Fig. 3C and 6). The *Planctomycetes* as a phylum, which contains anammox bacteria, were present at low levels in all samples and decreased in relative abundances in anoxic water in *Eurypon* sp. 2 and *H. stellifera* (see Fig. S6 in the supplemental material). In *Raspaciona* spp., conversely, the relative abundance of this phylum increases in anoxia from 2.4% to 4.7% (Fig. S6). The low signals of *Planctomycetes* in the sponges, other than *Raspaciona* spp., were likely contamination from microbes in the sponge water canal system and are usually bioinformatically filtered out of analyses of symbionts (for an example, see reference 90).

Curiously, both sulfate reduction and anammox bacteria have been confirmed in the holobiont *G. barretti*, which experiences internal anoxia in its tissues (49, 50). The difference between the microbial communities in *G. barretti* and those in anoxia-tolerant species from Lough Hyne might be due to differences in morphology, as *G. barretti* is a massive species, or environment, since it occurs more under constant oxygenation than under seasonal anoxia. Thus, the “anoxic microecosystems” observed in *G. barretti* (49) may result from its morphology, and the thin, encrusting sponges of Lough Hyne could be comparatively more oxygenated most of the year, even if pumping ceases (91). Periods of pervasive oxygenation would restrict symbioses with obligate anaerobes and favor microbes with flexible metabolic strategies.

Notwithstanding these potential anaerobic processes, the three symbiont combinations outlined above do not universally confer hypoxic tolerance to sponges in general but may be necessary for full anoxic tolerance. For example, *Thaumarchaeota* are effectively absent in the hypoxia-tolerant species *H. panicea* (92). Moreover, *H. panicea* contains high abundances (>75%) of an alphaproteobacterium as does its congeneric species *Halichondria bowerbankii* (Fig. 7), and *Alphaproteobacteria* were effectively absent from anoxia-tolerant Lough Hyne sponges. Although the microbial community within the hypoxia-tolerant *T. wilhelma* has not yet been investigated in detail, only two bacterial genomes have been identified from genomic sequencing of *T. wilhelma*, and both were likely *Alphaproteobacteria* (93). It is therefore unclear if *T. wilhelma* contains *Thaumarchaeota* OTUs in high abundance, although its congeneric species *T. citrina* does (Fig. 7). An alphaproteobacterium cultured from the Lough Hyne sponge *Axinella dissimilis* was able to grow anaerobically via fermentation and denitrification (94). Therefore, many microbiome structures may be capable of coping with hypoxia. Nevertheless, neither the hypoxia-tolerant *H. panicea* (17) nor *T. wilhelma* (16) tolerates

prolonged anoxia, so the Labhra Cliff holobionts may be uniquely tolerant to anoxia even in comparison to other poriferans. These sponge hosts may also be adapted to tolerate deoxygenation directly, or the ability to survive anoxia may depend on metabolic shutdown of either the host, symbionts, or both.

Potential adaptations of the sponge host to seasonal anoxia. It is unlikely that Lough Hyne sponges die off *en masse* during anoxia and recolonize the area during normoxia, because no dead tissue or discoloration was present around anoxic sponges. It is possible that these sponges decrease or cease metabolic activity during anoxia (25, 95), but this requires further investigation. For some marine invertebrates, environmental anoxia can trigger a switch to a fermentation-based metabolism, which results in a considerably decreased metabolic rate; however, the by-products of fermentation still need to be eliminated into the environment (reviewed in reference 95). If this elimination cannot be achieved by diffusion alone, it is possible that the sponges continue to pump during anoxia, albeit at a potentially decreased rate, and thereby still provide dissolved organics, ammonia, CO₂, and other metabolic products to their symbionts (Fig. 8).

Nonetheless, Labhra Cliff sponges were definitely pumping under hypoxic conditions (Fig. 1A), which is consistent with normal transcription activity in *T. wilhelma* under hypoxia (16) and with observations of sponges inhabiting consistently hypoxic environments (96, 97). Pumping was unsurprising considering that mobile fish and crabs were also observed under hypoxia at Lough Hyne (B. W. Strehlow, personal observation), and oxygen levels were above lethal and sublethal thresholds of many fish and invertebrates (12). Nevertheless, in a separate study, a single individual of *G. barretti* drastically reduced its pumping rate following *ex situ* oxygen depletion (20% air saturation) (98), and feeding rates in one individual (out of three) of *H. panicea* were reduced in low-oxygen concentrations (3% air saturation) (17). The sublethal, physiological impacts of deoxygenation, therefore, also need to be considered in the future.

Despite deoxygenation tolerance in some species, sponge diversity and abundance are overall likely limited by seasonal anoxia in Lough Hyne, and even *Eurypon* species and *H. stellifera* decreased significantly in abundance below the thermocline (20). Growth and reproduction may be impacted by seasonal anoxia because collagen synthesis is oxygen dependent; however, it is still possible in very low oxygen concentrations (see reference 99). Sponge larvae may also require elevated oxygen due to their motility. Also, elevated oxygen may be needed during settlement and early development in sponges; however, the specific oxygen requirements for these life stages remain unknown. Moreover, a combination of factors could restrict settlement and growth to fewer species. Although sedimentation rates in the anoxic region are equivalent to that of other sites in Lough Hyne (100), the combination of sediment and anoxic stress might restrict sponge distributions. Indeed, most encrusting species in Lough Hyne are more abundant on vertical than inclined (40°) surfaces due to high sedimentation on the latter (20). Additionally, seasonally decreased metabolic activity in the holobiont could limit growth or the production of secondary metabolites under anoxia, leading to increased spongivory in normoxic months when mobile predators return.

It is also possible that, like the sponges, some or all of the microbiome becomes dormant under anoxia. The capacity to become dormant is common and phylogenetically widespread in microorganisms (reviewed in reference 101) and may occur in response to environmental stress including hypoxia (102). For instance, pelagic *Thaumarchaeota* are present in sulfidic (anoxic) zones, but they exhibit lower expression levels of genes involved in ammonia oxidation and may be inactive (103). However, as stated in the previous section, *Thaumarchaeota* in symbiosis with sponges may possess elements of anaerobic metabolism (19) that could aid the sponge holobionts under anoxia if they are active. The major *Gammaproteobacteria* OTUs could similarly be inactive during anoxia in Lough Hyne, although there is also strong

evidence of anaerobic metabolisms within the clade formed by these OTUs within the anoxia-tolerant sponge holobionts. Moreover, even dormant microbes require some maintenance of proton motive force (102) or DNA repair (104). So, there may be some activity within a dormant microbiome under anoxia, which could perhaps be linked to the host's decreased pumping rate suggested above for waste elimination. Dormancy could also leave the holobiont vulnerable to predation by protists, but the dynamics of this potential interaction are unknown and warrant further study. If microbial dormancy occurs, it could still convey some resilience to the holobiont, stabilizing the microbiome under deoxygenation stress and allowing for rapid recovery following reoxygenation. However, this type of dormancy-recovery dynamic might be best suited for surviving seasonal anoxia, e.g., in enclosed or eutrophicated systems, whereas anaerobic metabolisms would be necessary for long-term survival in the functionally anoxic core of permanent OMZs.

Implications for early animal evolution and future oceans. These results have important implications for early animal evolution and the state of future oceans. Under the lower oxygen concentrations of the Neoproterozoic Era, early metazoans likely tolerated both widespread hypoxia and transient anoxia (105–107). The Labhra Cliff sponges demonstrated that this ancient tolerance is possible in sponges and that it could have involved the microbiome, but how analogous are these holobionts to early metazoans? The ancestral sponge evolved in a microbial world and may have consequently formed close associations with many symbionts like modern sponges. Modern symbioses with both *Gammaproteobacteria* and *Thaumarchaeota* were present in all 81 species assessed by Thomas et al. (40). It is therefore conceivable that the ancestral sponge contained either *Gammaproteobacteria*, *Thaumarchaeota*, both, or their respective ancestral forms. The loss or decreased abundances in these symbiont groups in sponge lineages that evolved into our “outgroup” samples (Fig. 7) could then correspond with the absence of this group in the seasonally anoxic site. The first metazoans could therefore have been very similar to the Labhra Cliff sponges in their symbiont composition and morphology, and even if it is only through convergent evolution, the seasonally anoxic sponges of Lough Hyne are an important model system for studying early animal evolution.

Considering the past also yields clues about the future. Some previous mass extinction events were probably driven by acidification, warming, and deoxygenation of the oceans following extensive volcanism (12, 108–111). Similar stressors are facing modern oceans as a result of anthropogenic CO₂ release. These emissions cause ocean warming, acidification, and the expansion of OMZs, and local deoxygenation can also be caused by anthropogenic nutrient runoff (1–5). For organisms like sponges and corals, benthic anoxia could stress adult life stages, while pelagic deoxygenation could threaten larval survival and distribution.

However, given the anoxic tolerances observed in sponges in the present study, could sponges outcompete corals in future scenarios (for examples, see references 112 and 113)? Sponge abundance has recently increased on some coral reefs, due in part to a decrease in coral abundance (114–116), but the future may be more nuanced. Recent experiments show that the necrosis and bleaching caused by thermal stress was ameliorated by increased CO₂ in two phototrophic sponge species under scenarios equivalent to the worst-case warming predictions, i.e., RCP8.5 (representative concentration pathway) (117), but necrosis was exacerbated by these two stressors in two heterotrophic species (118). Moreover, not all phototrophic sponges have this advantage, since at least one species died under these conditions (119), and most experiments do not include oxygen as a factor, which is predicted to decrease by up to 3.7% under RCP8.5 (120).

According to a meta-analysis across marine benthic organisms, the combination of thermal stress and deoxygenation reduced survival times by 74%, compared to each stressor in isolation, and increased the lethal concentration of oxygen by 16% on average (121). As with increased temperature and CO₂, responses to deoxygenation are

likely species specific, as suggested by this study. Some coral (122, 123) and sponge (11) species may be tolerant to deoxygenation, while others are not, and if anoxic tolerance in sponges is limited to seasonal exposure, the expansion of permanent OMZs could still cause mortality. The effects of the combination of all three stressors, however, are virtually unknown and thus require extensive research in the future.

Conclusions. This study demonstrated that distantly related sponge species can tolerate seasonal anoxia, and the cryptic diversity of sponges tolerating these extremes in Lough Hyne is high. The microbiomes of all sponge species were remarkably stable under varied oxygen conditions and mostly species specific, although some OTUs exhibited minor host sharing under anoxic conditions. Three different symbiont combinations were found to correspond with anoxic tolerance in Lough Hyne sponges, but future research is needed to verify the hypothesized metabolic interactions of host and symbionts under anoxia and to untangle microbial community structure from other shared characteristics. These microbial communities are not present in all sponge species with a reported hypoxic tolerance, but these combinations may be crucial for anoxic tolerance if only for their possible capacity for dormancy. The lack of significant disruption of the microbiomes during anoxia indicated that these sponge holobionts are well adapted to anoxia even without the HIF pathway. Similar sponge-microbe associations could have been important in the early evolution of animals under the lower oxygen conditions of the Neoproterozoic Era. Although our study suggests some strategies for holobiont deoxygenation tolerance, more research is needed to understand the individual and combined effects of acidification, warming, and deoxygenation to predict the structure of future ecosystems and to determine the cause-effect pathways of these stressors in order to inform management and restoration efforts.

MATERIALS AND METHODS

Study site. Sampling was performed in the Lough Hyne Nature Reserve (51°29'N, 09°18'W), a semi-enclosed marine lough in County Cork, Ireland. Lough Hyne is connected to the Atlantic Ocean by a narrow (~25-m), shallow tidal rapid. Most samples were taken from Labra Cliff (N51°30.0530' W9°18.1767'), located in an area known as the Western Trough (Fig. 1A). The water current speed at this site is $<5\text{ cm}^{-1}$, and the cliff surface, where sponges were found, is covered with a thick layer of silt (20). A seasonal thermocline exists and develops at ~25 m when surface temperatures increase in summer months. Below this depth, an anoxic/hypoxic layer forms (68).

Physical data. Oxygen concentrations and temperature were measured at increasing depths using a Pro20 dissolved oxygen meter (YSI, USA) (Fig. 1). Anoxic, hypoxic, and normoxic conditions were defined by dissolved oxygen concentrations of 0.00 to 0.01 (i.e., the detection limit of the instrument), 1.30 to 3.56, and 5.3 to 12 mg liter⁻¹, respectively. A conductivity, temperature, and depth instrument (CTD 90; Sea and Sun Technology, Germany), equipped with high-range (0.2 to 300 μM) and low-range (0 to 200 nM) oxygen sensors, was used to more accurately measure low concentrations of oxygen in July 2019. Temperature, turbidity, and salinity values measured from the CTD during July 2019 are reported in Fig. S1 in the supplemental material, while photosynthetically active radiation (PAR) and chlorophyll concentrations measured by the CTD are reported in Fig. S2A and B. The concentrations of photosynthetic pigments (chlorophyll *a* and pheophytin) were measured in July 2019 from water samples taken from 0 to 35 m at a distance of 5 m and at 39 m using a battery-powered pump (see reference 124) and as described in the supplemental material (Fig. S2C).

Sampling and DNA extraction. Sponges, water, and sediment were sampled at Labhra Cliff using scuba between July 2018 and August 2019 under permit no. R23-27/2018 issued by the Irish Department of Environment, Heritage, and Local Governments. A two-point calibrated HOBO dissolved oxygen logger (U26-001; Onset, USA) was used during all dives to measure dissolved oxygen (mg/liter) and to ensure that all sampled sponges were assigned to the correct oxygen condition, i.e., anoxic, hypoxic, or normoxic. Reference sponge samples were also taken above the 24-m depth, where oxygen is continuously present. The collection depths were recorded, and the oxygen condition of these samples was noted as "normoxic" (see Fig. 2 and Fig. S3 for full sampling details). Sulfide concentrations were measured from two 50-ml water samples at 27 m at Labhra Cliff and flash frozen in liquid nitrogen. After freezing, water samples were postfixed in 5% zinc acetate, and sulfide concentrations were measured spectrophotometrically in accordance with reference 125. The freezing may have degassed the samples, and some sulfide may have oxidized after the samples were collected but before they were frozen.

Sponges from the Labhra Cliff site were sampled using a flathead screwdriver to scrape off a ribbon of tissue that was then removed from the encrusted rock and placed into a Whirl-Pak bag using forceps. This process was optimized both to use minimal movement, limiting sediment resuspension, and to

ensure that divers stayed within no-decompression limits. Still, the depth and low visibility restricted the number of samples that could be taken, particularly during the anoxic period. All samples were frozen in liquid nitrogen soon after the divers reached the surface (a few minutes). Before sampling, the pumping status of five individual sponges under hypoxic conditions and five individuals under normoxic conditions was assessed visually using fluorescein dye. This procedure was not possible during anoxia due to time and visibility constraints.

Genomic DNA was isolated from sponge tissue (anoxic samples = 6, hypoxic samples = 25, normoxic samples = 39, total = 70) using the DNeasy (Qiagen, Germany) blood and tissue kit protocol. Sediment samples (hypoxic = 4, oxic = 3, total = 7) were extracted using a DNeasy PowerSoil Pro kit (Qiagen, Germany). Sterivex filters (Millipore, Billerica, MA, USA) were used to filter 1 liter of seawater (anoxic = 3, hypoxic = 4, oxic = 5, total = 12), the protocol from reference 126 was used to open filter casings, and a standard phenol-chloroform protocol (127) was performed to extract genomic DNA.

For microbial community comparison, “outgroup” samples were taken from three different species: *Halichondria bowerbanki*, *Suberites pagurorum* and *Tethya citrina* ($n = 3$ per species) from a separate location (51.499877, -9.296661) (Fig. 1A) at a depth of 2 m. These species were not observed at the seasonally anoxic site. For all sponge samples sampled from Labhra Cliff, less than 5% of the sponge volume was sampled, leaving sufficient biomass for survival and recovery (128).

Sponge identification. All sponge samples were identified to the genus and species level according to their phylogenetic position relative to known species by amplification of the *cox1* (~659 bp) gene using primers dgLCO1490 and dgHCO2198 (129) and the 28S (C region, ~550 bp) gene using primers C2 and D2 (130). Amplifications and cleanup followed the protocol described previously (131). The remaining supernatant was sequenced by MacroGen (Europe). BLAST searches against NCBI GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) were used to confirm sponge origin. Raw trace files were processed in Geneious v.11.1.5. Alignments were generated separately for each gene using the MAFFT plugin v.1.4.0 in Geneious with the L-INS-I algorithm (132), due to the heterogeneous taxon sampling and moderate sequencing success of *cox1*. Phylogenetic tree reconstructions were performed using the MrBayes 3.2.2 plugin on the CIPRES Science Gateway (133). The best fit evolutionary model (GTR+G+I) was selected according to the results of the JModelTest2 (134) plugin using the CIPRES Science Gateway v.3.3 (133). Two concurrent runs of four Metropolis-coupled Markov-chains Monte Carlo (MCMCMC) for 100,000,000 generations were performed and stopped when the average standard deviation of split frequencies was below 0.01. The first 25% of the sampled trees was removed as burn-in for further analyses. FigTree v1.4.2 (135) was used to visualize the trees.

Microbial community. (i) Library preparation. Sequencing libraries of *Bacteria* and *Archaea* 16S V4 rRNA genes were prepared based on a custom Illumina protocol (136) at DNASense ApS (Aalborg, Denmark). A detailed protocol for library preparation and purification is provided in the supplemental material (Text S1). Forward and reverse tailed primers were designed according to Illumina (2015) targeting the *Bacteria* and *Archaea* 16S V4 rRNA gene: abV4-C-f, GTGYCAGCMGCCGCGTAA; abV4-C-r, GGACTACNVGGGTWCTAAT (137, 138). Purified sequencing libraries were pooled in equimolar concentrations and diluted to 2 nM. Samples were paired-end sequenced (2×300 bp) on a MiSeq (Illumina, USA) at DNASense ApS using a MiSeq reagent kit v3 (Illumina, USA) in accordance with the standard protocol. Forward and reverse reads were trimmed and dereplicated, and processed reads were divided into OTUs and assigned relative abundances and taxonomies as outlined in the supplemental material (Text S1). The results were analyzed in R v.3.5.2 (139) through the RStudio GUI (140), using the ampvis2 package v.2.5.8 (141). All further analyses were performed using this package or base R unless otherwise indicated.

(ii) Statistical analysis. Samples from the sponges *H. stellifera* (anoxic = 2, hypoxic = 6, oxic = 1, total = 9) and *Eurypon* sp. 2 (anoxic = 2, hypoxic = 12, normoxic = 13, total = 27) as well as from water and sediment were subsetted due to their representative sampling under all three different oxygen conditions to form the “focused subset.” Variation in OTU composition within the focused subset was assessed by principal-component analysis (PCA) with Bray-Curtis dissimilarities. A canonical correspondence analysis (CCA) of OTU data constrained to the oxygen condition, using the Pearson chi-square measure and Hellinger transformation, was also performed on the focused subset to test for any influence of oxygen condition on microbial community structure. A CCA was also performed on all samples taken at Labhra Cliff ($n = 89$) as described above.

The presence of significant variation within the microbial communities based on sample type and oxygen condition within the focused subset was tested using a two-factor PERMANOVA with 10,000 permutations with the vegan package (142) in R. Significant differences in relative abundances were also tested between sample type and oxygen condition for the top seven most abundant OTUs individually within the focused subset, using analyses of variance (ANOVAs) for OTUs with normal distributions and Kruskal-Wallis rank sum tests for those with nonparametric data. Tukey’s honestly significant difference (HSD) tests and Wilcoxon pairwise tests were used for *post hoc* tests of parametric and nonparametric data, respectively. If one sample type across all treatments had zero percent abundance for a specific OTU, those data were excluded from analysis to meet assumptions of normality.

Separate bacterial and archaeal maximum likelihood phylogenies of partial 16S rRNA gene were calculated using RAxML v.8 (143) on the CIPRES Science Gateway V.3.3 (133). Alignments including the focused subset of the top bacterial OTUs (OTU4, OTU1075, OTU2, OTU5, OTU7, OTU6, and OTU17) and archaeal OTUs (OTU1 and OTU3) were merged with data sets of Feng et al. (43).

Data availability. All sample metadata, OTU table, alignments, physical data, and scripts (ampvis2) for this publication are available on github (https://github.com/astridschuster/Microbiome_MS_2020). The 16S sequence data are available under BioProject number PRJNA685112 in NCBI. Sponge barcode sequences were deposited at the European Nucleotide Archive (ENA) under accession numbers

LR880974 to LR881068 (<http://www.ebi.ac.uk/ena/data/view/LR880974-LR881068>) for 28S and LR880934 to LR880963 (<http://www.ebi.ac.uk/ena/data/view/LR880934-LR880963>) for *cox1*.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOCX file, 0.01 MB.

FIG S1, EPS file, 0.2 MB.

FIG S2, EPS file, 0.1 MB.

FIG S3, EPS file, 1.2 MB.

FIG S4, EPS file, 0.9 MB.

FIG S5, EPS file, 0.04 MB.

FIG S6, EPS file, 0.2 MB.

TABLE S1, DOCX file, 0.02 MB.

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We declare that we have no conflicts of interest.

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