Supporting Information

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Experiment 2: Sponge Survival Under the Continuous Flow of Degassed Seawater

A detailed description of our second experimental setup follows. This setup was primarily designed to determine the cause of the necrosis observed during experiment 1, as explained in the main text. Its main advantage is that it provides a constant supply of incoming seawater.

Toward the end of every run of experiment 1, the sponges developed visible necrosis, revealing the initiation of cell death. Each experimental run was terminated when the sponges looked more dead than alive (when roughly half of their visible surface was black with necrosis). Given this setup alone, it was difficult to determine whether the necrosis was triggered solely by the low levels of oxygen or by the accumulation of waste products, such as sulfide and ammonium, in the seawater. Likewise, it was unknown how the sponges would have looked if they had remained in the setup for the same duration under normal oxygen concentrations. For these reasons, we developed a complementary experimental setup that addresses these variables, as described below.

The additional setup (Fig. S2) involves two 1-L aquaria: one kept at 3–4% air saturation and one kept at 95–100% air saturation. Both of the aquaria are covered in tinfoil, and are supplied with a continuous flow of fresh unfiltered seawater via a peristaltic pump. The water entering the low-oxygen aquarium is first sparged with a mixture of N_2 gas and air, controlled by a gas mixer. The oxygen content of each aquarium is monitored with a FireStingO2, a USB-powered, fiber-optic oxygen meter. The health of the sponges was monitored qualitatively (primarily by color and firmness) and was photodocumented daily. Although this assessment is nonnumerical, it is fairly easy to visibly distinguish a healthy sponge from a dead sponge.

Two experimental runs were performed with this setup. During the first run, the sponge under low oxygen (roughly 3 cm across at the widest and 3 cm at the tallest) developed considerable necrosis, starting on day 9 after unintentional exposure to anoxia for a few hours, whereas the sponge under high oxygen (roughly 7 cm across at the widest and 3 cm at the tallest) was necrosis-free, even after 16 d (Fig. S3). The necrotic, low-oxygen sponge was reintroduced into a high-oxygen, uncovered tank. After 10 d, all visible necrosis was healed, although the sponge appeared to be more transparent and less massive, all while obtaining its original form (Fig. S5). The second run involved relatively smaller sponges (both roughly 3 cm at the widest and 3 cm at the tallest), with visibly higher surface area to volume ratios (numerous branching structures). During this run, both sponges, including the one under low oxygen, remained necrosis-free for the entire duration of the experiment, over 42 d in total, which totaled 24 d of <4% present atmospheric levels (PAL) for the low-oxygen sponge, due to numerous episodes of rising oxygen in between the replacement of nitrogen tanks. During this experiment, although no necrosis developed, small structural changes were visible in both sponges (Fig. S3), even during the <4% PAL intervals. In fact, the sponge under high oxygen underwent significant structural changes over the course of the experiment (Fig. S3). Comparable changes, however, were seen in most of the dozens of sponges kept in well-oxygenated holding tanks in the laboratory at this time.

These results demonstrate *Halichondria panicea*'s sensitivity to anoxia. However, any damage that does occur during prolonged exposure to anoxia is apparently reversible once normal conditions return. Tentatively, there appears to be a link between the size and internal surface area to volume ratio of a sponge and its sensitivity to low oxygen, although more research needs to be done to establish a definitive relationship. Nevertheless, it is reasonable that the size, morphology, and internal surface area of a sponge will influence its oxygen utilization and internal oxygen distribution.

Microbial Community

A rough estimate of *H. panicea*'s microbiome was made using terminal restriction fragment length polymorphism (T-RFLP) and sequencing of functional and 16S rRNA genes. Metagenomic DNA was extracted from 0.5 g of wet, nondried sponge tissue using the MO BIO PowerWater kit, following the manufacturer's protocol. For community fingerprints, 5–10 ng of extracted DNA were used in a 50 μL PCR containing 20 pmol of each: forward (B27F-FAM: [6FAM]5′-AGRGTTYGATYMTGGCTCAG-3′) and reverse (U519R: 5′-GWATTACCGCGGCKGCTG-3′) primers, 5 Units of Taq polymerase from Fermentas, and final concentrations of 40 μM dNTPs, 1× taq reaction buffer, and 0.5 mM $MgCl_2$. PCR conditions were as follows: 94 °C for 1 min followed by 28 cycles of 94 °C for 15 s, 54 °C for 45 s, and 72 °C at 45 s. The final elongation step was performed for 5 min at 72 °C.

For cloning Bacteria and Archaea 16S rRNA genes, we used the reverse primer U1492R (5′-GGYTACCTTGTTACGACTT-3′) combined with B27F (5′-AGRGTTYGATYMTGGCTCAG-3′) and A20F (5′-TTCCGGTGGATVCYGCCGGA-3′), respectively. Conditions were as follows: 94 °C for 1 min followed by 32 cycles of 94 °C for 20 s, 53 °C (50 °C for A20F) for 45 s, and 72 °C for 2 min. Final elongation was carried out at 72 °C for 5 min. Resulting PCR products were purified using the QIAEX II Gel Extraction kit from Qiagen following the instructions of the manufacturer. Purified PCR products were cloned into TOP10 competent *Escherichia coli* cells using the TOPO TA Cloning Kit for Sequencing from Invitrogen following the manual. Resulting clones were used for plasmid preparations using the GeneJET Plasmid Miniprep Kit from Fermentas following the instructions of the company. The final sequencing of the inserts was performed by Macrogen Europe (Amsterdam, Netherlands).

We analyzed 15 Archaea clones from the prepared 16S rRNA library and found all obtained sequences to be 98–100% identical to each other. Using the Basic Local Alignment Search Tool (BLAST), located on the webpage of the National Library of Medicine, we found these sequences to be 98–99% identical with sequences from *Candidatus* Nitrosopumilus koreensis, a known ammonium oxidizing archaeon. Additionally, obtained archaea *amoA* sequence showed 91% similarity to the *amo*A sequence found in *Candidatus* Nitrosopumilus. Therefore, these preliminary results suggest the presence and relatively high abundance of ammonium oxidizing archaea in *H. panicea*.

The community fingerprint for the bacterial community revealed 14 operational taxonomic units (OTUs). However, five of these OTUs seem to form the majority of the bacterial community (Fig. S6). We furthermore analyzed 15 sequences obtained from the bacterial 16S rRNA library using the BLAST tool (Table S1). The sequences, which were the most similar to the obtained bacterial sequences, are affiliated with oxic environments and likely belong to aerobic bacteria. Based on these findings, it is likely that the bacterial community is dominated by aerobic metabolisms. This is corroborated by the obtained bacterial *amoA* sequence from the sponge community. This sequence was found to be 96% identical to the *amo*A sequence from an uncultured betaproteobacterium. Ammonium oxidation is therefore likely to be a common aerobic microbial metabolism in *H. panicea*.

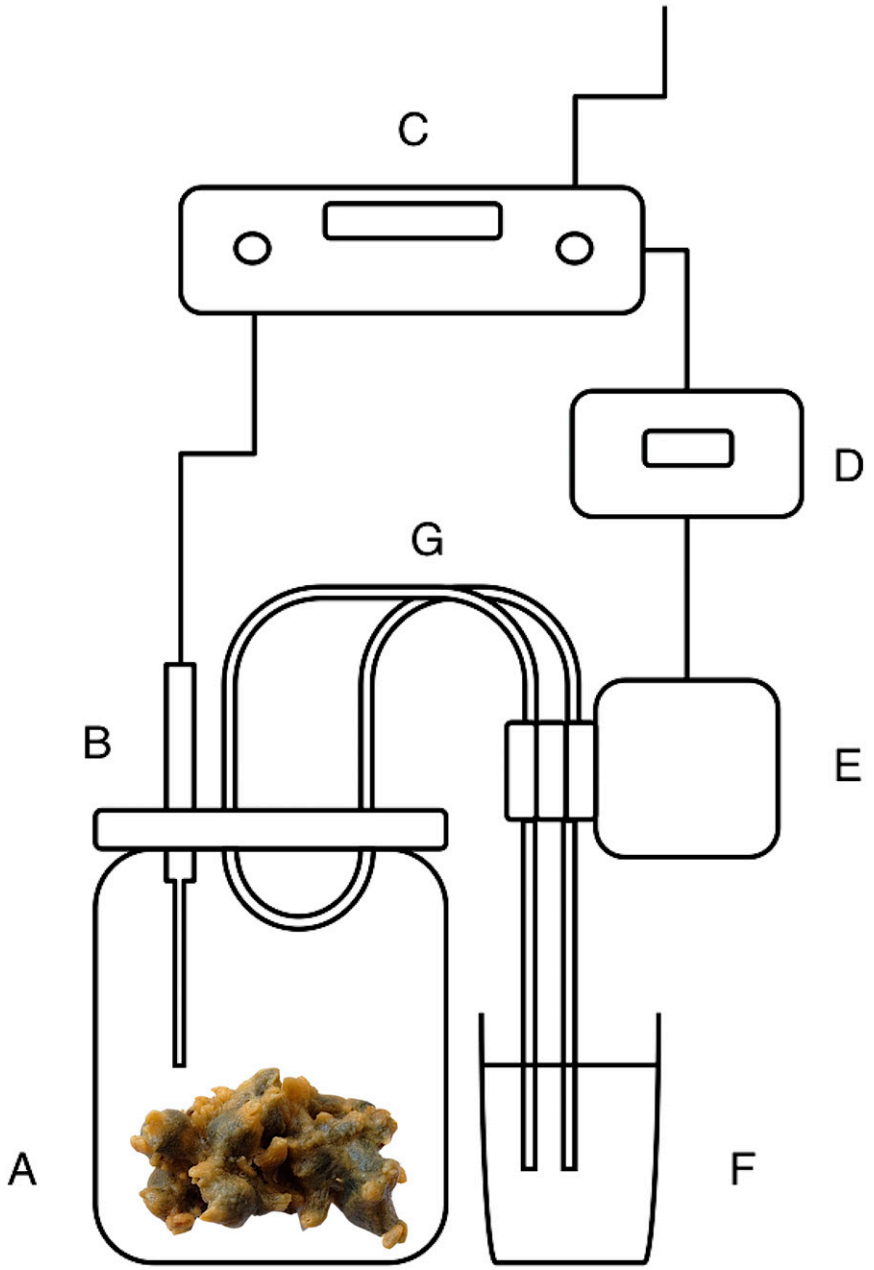

Fig. S1. Setup from experiment 1: a sponge occupies an airtight 1-L container filled with seawater (indicated by A); a microelectrode (indicated by B) constantly measures the oxygen concentration in the seawater, outputting the signal to a picoammeter (indicated by C); as oxygen is consumed via sponge respiration and falls below the set limit, a regulator (indicated by D) turns on a pump (indicated by E), cycling aerated water (indicated by F) through silicone tubing (indicated by G) running through the closed container. Oxygen diffuses out of the tubing into the container, raising the oxygen concentration of the water. Once oxygen rises above the set amount, the pump turns off, allowing the sponge to draw down oxygen again.

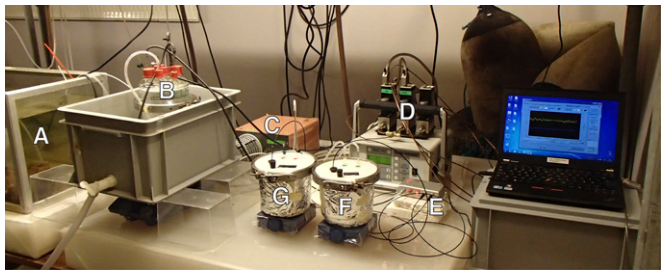

Fig. S2. Nonregulatory setup with continuous water flow. Holding tank where incoming seawater is introduced (indicated by A); sealed reactor where fresh seawater is sparged with the gas mixture (indicated by B); peristaltic pump that transfers the water from the holding tank to sponge aquaria (and the sparging reactor for the low-oxygen aquarium) (indicated by C); gas mixer (indicated by D), combining a mixture of N_2 gas and air used to create 3.5% air saturated water; FireStingO2 fiber-optic oxygen meter (indicated by E); high-oxygen sponge aquarium (>95% air saturation) (indicated by F); and low-oxygen sponge aquarium (3–4% air saturation) (indicated by G).

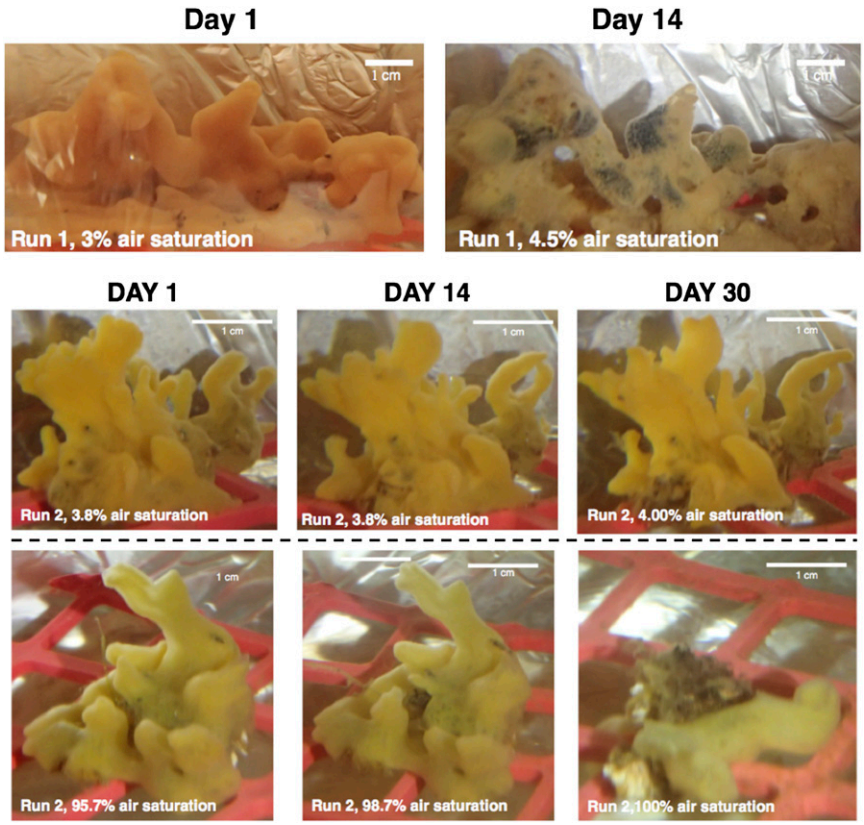

Fig. S3. A comparison of three sponges from two different runs. The low-oxygen sponge from run 1 (*Top*) is compared with both the low-oxygen (*Middle*) and high-oxygen (*Bottom*) sponges from run 2.

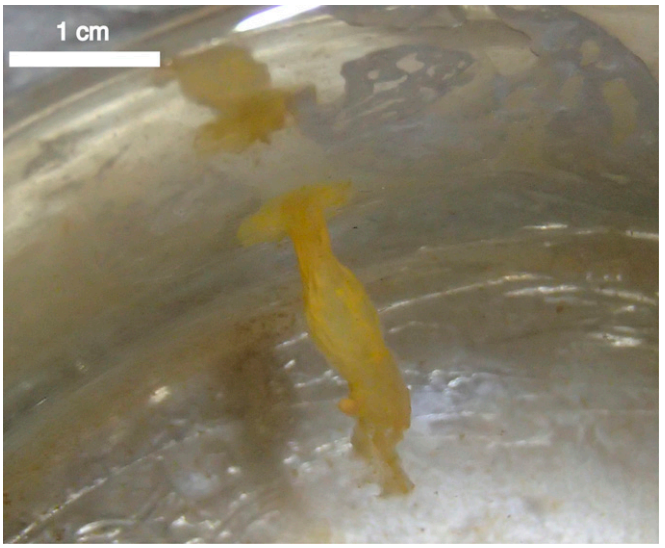

Fig. S4. An appendage from run 2's low-oxygen sponge attached to the side of the aquarium, revealing active growth under low-oxygen conditions.

Fig. S5. The low-oxygen sponge from run 1. (*Upper*) After 16 d under low-oxygen conditions (3–4.5% air saturation). (*Lower*) Ten days after being returned to a high-oxygen environment (100% air saturation).

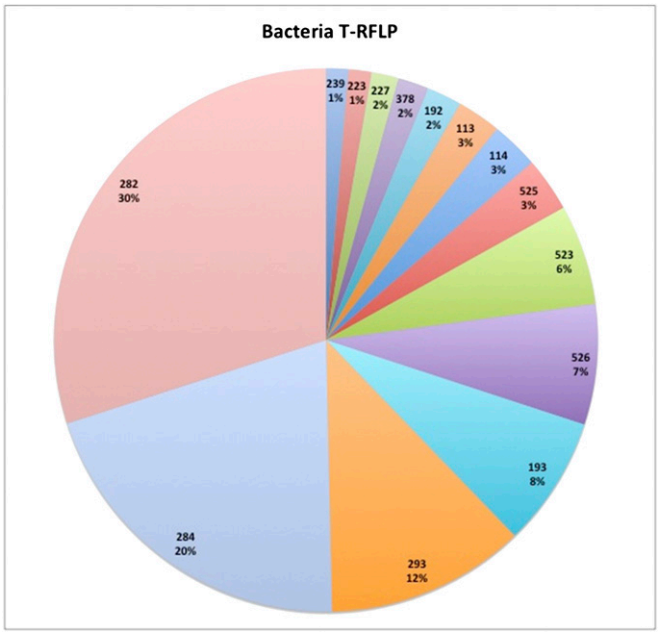

Fig. S6. T-RFLP results based on bacterial 16S rRNA, restricted with *Bsu*RI. Numbers designate terminal fragment size (base pairs), and percentages represent relative abundance.

Table S1. Bacterial 16S rRNA BLAST results

Clone	Description	Sequence query cover, %	Max identity, %
SB01	**Uncultured Bacterium**	**87**	**91**
SB02	Uncultured Bacterium	100	99
SB03	Uncultured Bacterium	94	100
SB04	Uncultured marine Bacterium	99	99
SB06	**Uncultured planctomycete**	**91**	**91**
SB07	**Uncultured Bacterium**	**98**	**95**
SB08	*Loktanella* sp.	99	98
SB09	**Uncultured marine Bacterium**	**100**	**92**
SB10	**Uncultured marine Bacterium**	**99**	**88**
SB11	Uncultured Myxococcales	100	99
SB12	**Uncultured alpha proteobacterium**	**100**	**92**
SB13	Uncultured *Xenococcus* sp.	94	99
SB14	Uncultured Cyanobacterium	99	97
SB15	**Uncultured Methylophilaceae**	**99**	**89**
SB16	Uncultured actinobacterium	99	99

Bold indicates hits that are <97% identical.