Assessing the utility of sponge microbial symbiont communities as models to study global climate change: a case study with *Halichondria bowerbanki*

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**Abstract:** We examined the stability of sponge microbial communities in *Halichondria bowerbanki*, a common sponge in the Chesapeake Bay (Virginia, USA), in response to potentially stressful thermal environments. Sponges were reared under three thermal regimes that corresponded to current thermal maxima and realistic projections of SST increases over the next 50 and 100 years. No obvious changes in the density or diversity of microbial populations were observed when electron micrographs were compared among treatments. However, denaturing gradient gel electrophoresis (DGGE) uncovered consistent changes among the three treatments. Some bands obtained via DGGE disappeared when sponges were reared under thermally stressful temperatures indicating loss or significant reduction in population size of some species. Some bands were present only in the warmest treatment, which may indicate that a rare species increased in relative frequency in the microbial community. Sponge microbial communities offer numerous opportunities to explore hypotheses generated by current community ecological theory. We are only beginning to understand the significance of these sponge-microbe associations in the context of global warming.

**Keywords:** community stability, global warming, microbial symbionts

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**Introduction**

Among eukaryotes harboring prokaryotic symbionts, some marine sponges stand apart for harboring extraordinarily dense and diverse communities that are composed, at least in part, of a derived, sponge-specific microflora (e.g., Lee *et al.* 2000, Hentschel *et al.* 2003, Hill 2004, Hill *et al.* 2006). It is not uncommon for the symbiont community to occupy a larger volume than that of the host cells (Santavy *et al.* 1990, Brantley *et al.* 1995). The symbionts appear to perform vital functions for their hosts and perhaps the entire benthic community (e.g., nutrient cycling (Corredor *et al.* 1988) and production of secondary metabolites (Piel 2004)). These ancient symbioses can involve prokaryotic and/or eukaryotic partners, and can occur in intra- and extracellular locations (Wilkinson 1987).

Despite the evolutionary and ecological significance, we know relatively little about how these associations are structured and how stable these communities are in the face of environmental change (but see Vicente 1990, Cerrano *et al.* 2001). While there is a wealth of information on the response of corals and their zooxanthellar symbionts to stressful conditions (e.g., Hoegh-Guldberg 1999), only a handful of studies have examined the stability of symbiont communities in sponges as they respond to environmental factors (e.g., Vicente 1990, Friedrich *et al.* 2001, Thoms *et al.* 2003, Taylor *et al.* 2004). There are often few clear phenotypic markers that could be used for visual assessment of association health for sponges that lack symbiotic phototrophs. Thus, we have only limited ideas about what kinds of changes may be occurring between sponges and their symbionts during stress inducing events. This ignorance represents an unfortunate state of knowledge because changes may occur that have important consequences for sponge health and ultimately community structure.

Survey-based analyses have found that some sponge-bacterial symbioses appear to be stable across large spatial scales despite including hosts that belong to different orders and occupy highly dissimilar habitats (e.g., Hentschel *et al.* 2002, Taylor *et al.* 2004, Webster *et al.* 2004, Olson and McCarthy 2005, Hill *et al.* 2006). Furthermore, some laboratory and field experiments have found sponge-bacterial symbioses to be remarkably stable. Friedrich *et al.* (2001) found that antibiotics and starvation had little effect on the symbiont communities present in *Aplysina aerophoba* reared in aquaria. Thoms *et al.* (2003) transplanted Mediterranean *A. cavernicola* from 40 m to depths as shallow as 7 m and found that bacterial communities harbored by the sponge were largely unaffected by the novel environmental conditions. Not all sponges, however, harbor immutable bacterial communities. *Cymbastela concentrica* exhibited limited, short-term changes in microbial communities over small
geographic distances. Furthermore, the microbial community present in temperate populations of *C. concentrica* was distinct from the community existing in tropical populations of this species (Taylor et al. 2004, 2005). These studies highlight the need for additional work in the analysis of sponge-associated microbial diversity. This is especially true for temperate sponges, which remain very poorly studied (but see Wichels et al. 2006) and will also face significant increases in sea surface temperature (SST - McCarthy et al. 2001).

The Third Intergovernmental Panel on Climate Change (IPCC) found that the Earth's average temperature had increased by \( \approx 0.8^\circ \text{C} \) since the start of the 20th century and may exceed an increase of \( 2^\circ \text{C} \) by 2050 (McCarthy et al. 2001, Thomas et al. 2004). In marine systems, projected increases in SSTs over the next 100 years indicate that stressful conditions will intensify in many marine habitats and that habitat deterioration will become more apparent (e.g., Hoegh-Guldberg 1999, Knowlton 2001, Walther et al. 2002, Bellwood et al. 2004, Sheppard and Rioja-Nieto 2005). For example, relatively small (\( \approx 0.1^\circ \text{C} \)) increases in average SST correlate strongly with increases in the amount of bleached coral cover while mass bleaching events occur when SST anomalies exceed only \( 0.2^\circ \text{C} \) (McWilliams et al. 2005). Models indicate that SST will increase globally up to \( 1.2^\circ \text{C} \) over the next 50 years and \( 2.4^\circ \text{C} \) over the next 100 years (McCarthy et al. 2001, Wigley and Raper 2001). These temperature increases are not confined to the surface but can penetrate to \( > 500 \text{ m} \) (Barnett et al. 2005). The consequences of \( 1^-2^\circ \text{C} \) increases for marine fauna of non-tropical regions can be quite severe. In 1999, the Mediterranean experienced temperature increases in this range that lead to mass mortality of gorgonian and sponge populations in the Ligurian Sea (Cerrano et al. 2000, 2001, Perez et al. 2000). Our ignorance of the ecological effects of climate change of the sort predicted may have substantial ecological consequences.

The goal of this work was to begin to assess changes in the bacterial community harbored by the temperate sponge *Halichondria bowerbanki* when exposed to elevated temperatures of extended duration. We argue that this system could serve as a model for marine ecosystems for a number of reasons. The complexity of the symbiotic community provides opportunities to explore whether relatively small changes in environment can modify ecological relationships among competing species (e.g., Jiang and Morin 2004). The often important physiological role of symbiotic species provides an opportunity to examine the consequences of environmental change on symbiotic, and therefore host, performance. Finally, the sponge-symbiont association provides a tractable system and therefore permits experimentation in a manner that is exceedingly difficult if not impossible for many other communities.

**Materials and methods**

**Sponge collection**

*Halichondria bowerbanki* individuals (\( n = 3 \)) were collected via snorkeling from a pier at the mouth of the York River in the Chesapeake Bay (Lat. +37.246, Long. -76.500). Collections were made near the mean low water line during the end of July 2005. Sponges were immediately transported to the laboratory in aerated containers and processed within 4 h.

**Experimental design**

Sponges were reared in separate 1-liter containers that were part of a re-circulating seawater system with a 100 L reservoir tank (\( \approx 125 \text{ L total system volume} \)). Three samples (\( \approx 1 \text{ cm}^3 \)) from each sponge were placed in each container. Temperature conditions for the containers were assigned randomly. Control containers (\( n = 7 \)) were set at the thermal maximum experienced at the collection site (\( \approx 29^\circ \text{C} \)). This temperature was achieved by heating the reservoir tank to the desired temperature via a submersible heater. After passing through the heated system, the water was diverted to a chiller that cooled the water to room temperature before it returned to the reservoir tank. Treatment chamber temperatures were either \( 1^\circ \text{C} (n = 9) \) or \( 2^\circ \text{C} (n = 7) \) above the thermal maximum. Individual heaters were placed in the containers to achieve the desired temperatures. Temperature profiles were stabilized before sponges were placed in the system. During the 14 d experiment, approximately one third of the volume of the entire system (\( \approx 40 \text{ liters} \)) was exchanged with fresh Bay water every other day to regulate salinity, remove waste products, and provide sponges with natural bacterioplankton communities. Water temperature, salinity and overall sponge health were monitored twice daily for the 14 days of the experiment. Small adjustments to heaters were made as required to keep temperatures at the desired level.

**Molecular analysis**

At the conclusion of the experiment, two samples from each replicate container were immediately frozen at \(-80^\circ \text{C} \) for subsequent molecular work. DNA was isolated from sponge samples that had both choanoderm and pinacoderm using a standard CTAB isolation protocol (Hill et al. 2004). DNA was quantified and diluted to 50 ng µl\(^{-1}\). Regions of small subunit rDNA were amplified using two sets of primers. The first set of primers (1055f and 1406r; Webster et al. 2004) amplified a 350bp region conserved in the domain *Bacteria*. The second set of primers (PRBA338f and PRUN518r) were taken from Øvreås et al. (1997) and amplified approximately 180bp of a more variable region and were used to sample a wider taxonomic range of sponge-associated microbes.

Promega’s GoTag® reagents were used for all PCR reactions. Ten pmol of each primer and 50 ng of DNA were used in each reaction. The GoTag® master mix yielded final concentrations of 1.5 mM and 2.5 mM for MgCl\(_2\), and dNTPs respectively when diluted with ultra-pure water. We typically ran 20 µl final volume reactions. The PCR program included an initial denaturation run of 2 min at 95°C. This was followed by 35 cycles of the following profile: 1 min at 95°C, 30 s at 57°C (for 1055f and 1406r) or 30 s at 67°C (for PRBA338f and PRUN518r), and 45 s at 72°C. This was followed by a final extension of 6 min at 72°C. PCR products were run on a 1% agarose gel to examine the quality of amplification. To compare profiles of the bacteria found in sponges reared at different temperatures, the Bio-Rad Dcode® Universal
Mutation Detection System was used for Denaturing Gradient Gel Electrophoresis (DGGE).

A 1 mm thick, 10% (w/v) polyacrylamide gel with a 30-60% formamide:urea denaturing gradient was poured following manufacturer’s instructions. Electrophoresis was performed in a 1X TAE buffer solution at 33V for 16 hours for PCR products derived using primers 1055f and 1406r. For products obtained using primers PRBA338f and PRUN58r, gels were run at 44V for 18 hours. All gels were stained with ethidium bromide and images were captured using UV transillumination on the Kodak Gel Logic® camera system.

Microscopy

For electron microscopy, sponge samples from each treatment were fixed in 2.5% glutaraldehyde and sterile filtered seawater. Samples were washed in 0.2 M cacodylate buffer (pH 7.4) and postfixed with 1% OsO₄ and 1% Uranyl acetate. Samples were then dehydrated in an ethanol series, infiltrated in propylene oxide, and embedded in Embed 812 plastic resin. After polymerization, 1 mm sections were cut and treated in 4% hydrofluoric acid:76% ethanol to dissolve spicules. These sections were then dehydrated, infiltrated, and embedded again following the protocol described above. Ultrathin sections were stained with uranyl acetate and quick lead. Micrographs were taken using a Joel transmission electron microscope.

Results

Average (±SEM) temperatures during the course of the experiment were as follows: control (28.6 ± 0.04 °C), +1°C treatment (29.8 ± 0.04 °C), and +2°C treatment (31.3 ± 0.04 °C). Temperature variability observed during the course of the experiment arose primarily as a function of fluctuations in building temperature and daily values for each treatment are shown in Fig. 1. Regression analysis indicated that there were no differences in temperature among days within treatments (i.e., slope of the line was 0, Fig. 1). Analysis of variance indicated significant differences among treatments (p < 0.0001; F²,226 = 926.06) and post hoc Tukey’s HSD indicated that each level was significantly different from the others. There were no significant differences among days within the controls or +2°C treatment (p > 0.28, F⁹,₆₀ = 1.58 and p > 0.14, F⁹,₆₀ = 1.58 respectively). Although a significant difference was found among days within the +1°C treatment (p = 0.025, F⁹,₇⁹ = 2.29), post hoc Tukey’s HSD found no significant differences among days. Implementing Hsu’s Best MCB post hoc test indicated that days 2 and 6 were significantly different than days 1, 8, and 13.

A fraction of the bacteria associated with Halichondria bowerbanki was found to be sensitive to the temperature changes experienced in these experiments. DGGE banding patterns differed between treatment and control sponges for the PCR reactions that used primers 1055f and 1406r (Fig. 2). At least three bands present in the control treatments were consistently lost in the +1°C and +2°C treatment levels (arrowheads on the left, Fig. 2). Two bands discovered in the +2°C treatment were not present in the control or in the +1°C treatment (arrowheads on the right, Fig. 2). Comparisons between the sample sponges and samples of the source sponge taken at the time of collection were not performed for this primer set.

Because the PRBA338f and PRUN518r primers amplify a more variable region of 16S rDNA, the banding patterns obtained were more complex than those observed above. However, as in Fig. 2, we found evidence of the loss and gain of bands in the +2°C treatment when compared to the other temperature conditions (see white and black arrowheads in Fig. 2: PCR-Denaturing Gradient Gel Electrophoresis performed with primers 1055f and 1406r. The arrows on the left identify species that may be sensitive to the temperature treatments employed in these experiments and were subsequently lost from the symbiont community. The arrows on the right identify two bands that were only present in the warmest temperature treatment.)
Fig. 3). We also found some evidence of aquarium effects because one band was only present in samples taken from the source sponges at the time of collection (star on the left, Fig. 3). Another band was found in the majority of the heated treatments, but was also found in one control sponge (star on the right, Fig. 3).

Bacterial densities were found to be low in *H. bowerbanki* (Fig. 4A-C). However, five distinct phenotypic types were identified in this study. The first type (Fig. 4D) had thin projections that extended in some cases > 0.5 µm from the surface of the bacterium. Another type (Fig. 4E), had projections that resembled small bumps on the surface of the bacterium, which were uniformly spaced at an interval of ≈ 0.16 µm. Another commonly observed microbe was cylindrical to ovoid in shape and had a non-uniformly electro-dense cytoplasm (Fig 4F). A rod shaped type was also observed that had electro-dense inclusions in its cytoplasm (Fig. 4G). Finally, a cylindrical to ovoid bacterial type was identified that appeared to be encircled by a collagen layer and was observed to have a nuclear region that was indistinct and fibrous (Fig. 4H).

**Discussion**

This research represents one of the first attempts to study changes in sponge microbial symbiont communities that might be expected if sea surface temperatures (SSTs) increase as predicted under conditions of global warming. The treatment levels employed in these experiments were chosen to reflect conditions under conservative models of SST warming. During the experiment, our goal was to keep temperatures below 20% of the mean and we largely achieved this for the +2°C treatment and the control. One of the problems we faced in these experiments was maintaining the +1°C treatment levels so that they did not overlap with either the control or the +2°C treatment. In one instance, a replicate in the +1°C treatment approached the mean temperature for the controls. In another instance, a replicate in the +1°C treatment exceeded the average temperature for the +2°C treatment; two other replicates also came close to that average. The significant differences recorded using Hsu’s MCB can be explained by the latter data. Nonetheless, significant differences recorded among treatment levels, and lack of overlap within the 95% confidence interval, indicate that we largely achieved the desired temperature separations. However, in future experiments, we will work to control the precision within and separation between treatments. Due to their location in shallow water habitats, however, daily fluctuations in temperature are likely to be as extreme as was encountered in this experiment.

Results indicate that the sponge symbiont community changes in response to conditions that mimic 50-100 year projections of sea surface temperature increases. Analysis of DGGE gels revealed segments of the symbiont community that are negatively affected by increases in temperature (Figs. 2 and 3). These bacteria may be lost from the community (a local extinction) or may decrease to such low densities that we were unable to amplify their DNA. Regardless, the loss of species under the types of controlled conditions described here would provide an important opportunity to study in detail ecological consequences of reductions in species richness that are predicted under various global warming scenarios.

Furthermore, our data indicate that some species of bacteria harbored by sponges may increase in frequency under the highest temperatures (Figs. 2 and 3). It is important to determine whether these bacteria begin at lower frequencies (below the threshold easily detected with DGGE) and then gain a competitive advantage under the new temperature environment or are acquired from the environment. Given limitations in DGGE analysis to monitor changes in relative frequencies, we are exploring other options (e.g., FISH) to follow community level parameters of species richness and relative abundance. We are also beginning to obtain sequences from the variable bands so that we may begin to identify the species that are susceptible to this type of environmental change.

As has been found in other studies examining changes in sponge symbiont microbial communities, morphological characteristics proved of limited value in attempts to chart
changes in species richness. The morphological diversity discovered in *H. bowerbanki* appears similar to that identified in other temperate sponges. While it was impossible to determine which bacterial types were affected by the thermal conditions devised in these experiments, some interesting types were uncovered. We are particularly interested in identifying species with projections (Fig 4D and E) as these types have been observed in other sponges (M. Maldonado, pers. comm.).

Greater work needs to be done to more fully understand the consequences of warming sea surface temperatures in the context of sponge microbial symbiont communities. The work presented here demonstrates that controlled experimental conditions can be created to mimic proposed increases in seawater temperatures and community profiles can be monitored using molecular tools. What is needed is a more precise determination of which species of symbiont are susceptible to temperature changes and the community-wide consequences of these shifts in community composition and structure. We are currently working to answer these types of questions.

Acknowledgements

We thank the Virginia Institute of Marine Science, Gloucester Point, Virginia for permission to collect on their pier. Carolyn Marks’ guidance with electron microscopy is greatly appreciated. Comments from two reviewers were especially helpful.

References


