JOURNAL OF
EMERGING INVESTIGATORS

Article

The effects of stress on the bacterial community associated with the sea anemone *Diadumene lineata*

Rebekah Cahill[1], Stacy A. Krueger-Hadfield[2], Will H. Ryan[2]
[1]Jefferson County International Baccalaureate School, Birmingham, Alabama
[2]Department of Biology, University of Alabama at Birmingham, Birmingham, Alabama

SUMMARY

The bacteria found on and inside animals can play important roles in the health and function of the host individual. However, the factors that control which species colonize a host are complex. In this study, we investigated the effect of environmental stress on the composition of the bacterial community associated with the sea anemone *Diadumene lineata*. We grew anemones in both normal and stressful conditions and then cultured bacteria from each individual anemone and its mucus separately on R-2A agar. We found that anemones grown in normal conditions had microbiomes that were distinct from the surrounding seawater, but that the community of bacteria found on stressed anemones was more similar to that found in the seawater. Interestingly, *D. lineata* produces a thick mucous covering when stressed, which may play a role in controlling the microbial community during stressful conditions. We found that the mucus had a distinct community of bacteria with very few taxa present. These results were consistent with findings from corals and other sea anemones that suggested that stressed hosts are less able to control the microbes that colonize their surfaces and that mucus may have antimicrobial properties that influence microbial community assembly. The findings of this study support the use of sea anemones for further investigation of the complex interaction among hosts, microbes, and their environments. Our study also highlights the need to investigate sea anemone mucus further, both as an ecologically important substance and as a potential source for novel pharmaceutical compounds.

INTRODUCTION

As the tools available to study microbes have improved, we have become better able to appreciate the role that microbes play in the health of organisms and ecosystems. It is now understood that bacteria and other microscopic organisms can form close associations with plants and animals and contribute to their health and function (1). In addition, the microbes associated with common plants and animals, generally called the microbiome, can be an important source of new chemicals that may have properties useful to humans (2). Thus, characterizing the microbes associated with plants and animals is an important goal for both basic and applied sciences.

The relationship between a macroscopic host organism and its microbiome is complex. The environment created by the host may control what species of bacteria can thrive nearby. The bacteria can also have a positive or negative effect on the host (e.g., gut microbes aiding in digestion or bacteria causing illness). The bacteria that are capable of colonizing a host depends on the species available in the local environment, and how those species interact with the host organisms (3). Emerging evidence suggests that organisms tend to show a consistent and intimate relationship with a small core set of bacterial types among the hundreds or thousands of species that can be found occasionally on or inside an individual (4). Different parts of the same organism can maintain different microbiomes. For example, the microbiomes of coral tissue and the layer of mucus covering the tissue have been shown to be distinct (4). The composition of both the core and ephemeral bacterial communities that thrive on or near a host can also depend on the environment in which the host is found, as each bacterial species has its own set of requirements for life that must be met before it can grow.

In this study, we measured the influence of the external environment on the community composition of microbial species associated with the sea anemone *Diadumene lineata*. We chose this species because it can live in a wide variety of environments, which may help us understand the role the external environment has in shaping the microbial community. The anemone *D. lineata* is thought to be native to the Northwest Pacific Ocean, including the coasts of Japan and China. However, the species has successfully invaded coastal waters across a variety of regions around the world including the Pacific, Atlantic, and Gulf Coasts of the United States (5). Its documented ability to tolerate weeks of exposure to temperatures between 3° and 29°C and salinities ranging from near freshwater to a high salinity of at least 34 parts per thousand have likely contributed to its success as a widespread non-native species (6,7). *D. lineata*'s status as an invasive species and its broad environmental tolerance make it an interesting subject for studying how the interaction of the environment, the locally available pool of species, and the anemone's physiology shapes the diversity of its microbiome.

Cnidarians, like corals and sea anemones, also have interesting microbiomes because of the properties of the mucus they produce to protect their tissues. The chemical properties of cnidarian mucus appear to influence the composition of bacteria that can colonize these species (4).

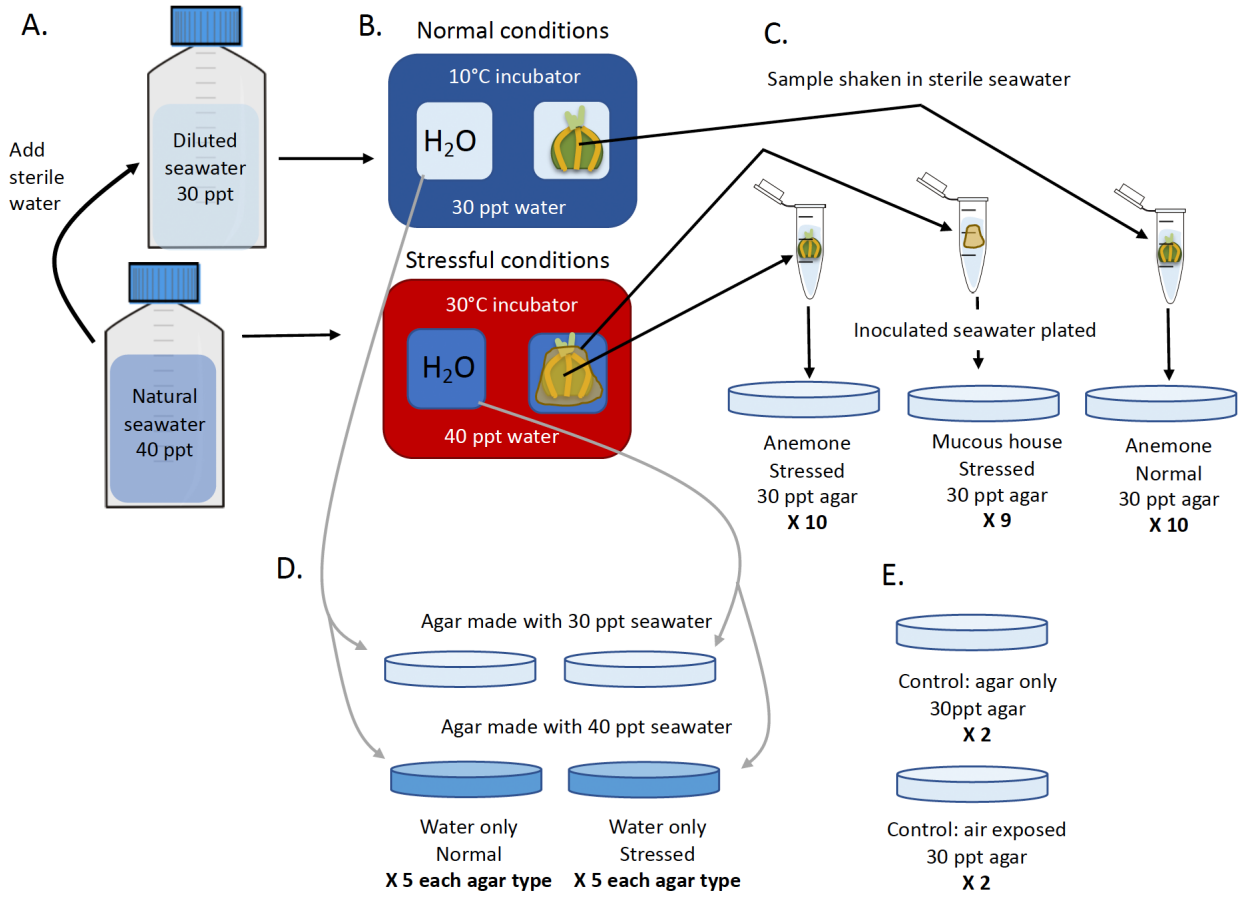

Figure 1. Experimental design. (**A**) Natural, unsterilized seawater was diluted with sterile DI water. (**B**) High and low salinity water were then distributed to plates and placed in to a high and low temperature incubator respectively. The treatment combination of high salinity and high temperature was called "stressful," whereas the low salinity, low temperature treatment was called "normal." (**C**) After two weeks, anemones were removed from each well and placed in a tube of sterilized seawater. Where mucous houses were produced (stressful treatment), the mucous house was placed in a separate tube of sterile seawater. The tube was shaken vigorously to inoculate the seawater with bacteria from the anemone and plated on normal agar. (**D**) An equal amount of water was taken from each of the water-only wells in each treatment and plated individually on agar plates of either normal or high salinity. (**E**) Two sets of control plates were established to ensure that all bacteria observed came from the seawater or anemones. Two normal salinity agar plates were kept sealed to check for contamination in the agar. Two normal salinity agar plates were exposed to the air for a short period to check for contamination from the air.

For example, mucus from the anemone *Actinia equina* has been shown to have hemolytic, cytotoxic, and antibacterial activity in laboratory studies (8). Of interest to our study, *D. lineata*, is known to respond to environmental extremes by secreting a thick mucus coating and remaining sealed inside the structure (hereafter called a "mucous house") until conditions improve (9). It is unknown what role the production of this mucous house has in protecting the sea anemone from harm, but we predicted that the production of such a structure may help protect the sea anemone from harmful changes in the anemone's microbiome, as the host tissue becomes more susceptible to infection under stress.

To better understand the nature of the relationship among the host anemone, mucus, and microbiome, we tested three hypotheses about the factors influencing the microbial diversity associated with this sea anemone. First, the microbiome of the anemone will differ from the seawater surrounding it, second, the community of bacteria associated with the sea anemone will differ under stressful versus normal culture conditions, and third, the bacteria associated with the mucous house will differ from both the seawater and the live anemone tissue.

RESULTS

To understand the role of the external environment on the community of bacteria that are associated with the sea anemone *D. lineata*, we exposed replicate sea anemones and seawater samples to either normal or stressful culture conditions in the laboratory (**Figure 1**). After seven days in culture, we characterized differences in the microbial community between treatment by growing bacteria associated with the seawater, anemones, and mucus produced by the anemone on agar.

Because our methods did not allow us to diagnose the specific bacterial species in the study, we referred to the visually unique colony types as Operational Taxonomic Units (OTUs). Each OTU was assigned an arbitrary letter (A through O), as we were unable to identify bacterial species in

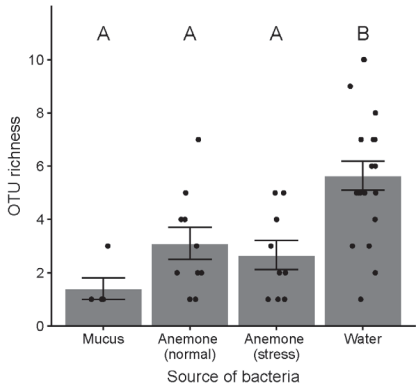

Figure 2. Mean ± standard error OTU richness by source of bacteria. A one-way ANOVA showed that there was a significant difference between groups ($F_{3,40}$ = 8.68, p = 0.001). Tukey HSD post-hoc analysis showed that the water samples had significantly more OTUs on average than the mucus or either type of anemone samples. Different letters above bars denote groups that have significantly different mean OTU richness (pairwise p value between groups unalike letters is less than 0.05). Points are jittered slightly to reduce overplotting.

this study. On average, 3.9 ± 0.39 SE OTUs were found per plate.

To distinguish changes due to the interaction of the anemone and the environment from changes in the bacterial community available in the water due to the experimental conditions, we also characterized the bacterial community in replicate samples of seawater exposed to each treatment condition described above in the absence of sea anemones. We found no effect of either culture environment or agar type on the OTU richness in the water-only samples (Condition: $F_{1,16}$ = 0.01, p = 0.927; Agar: $F_{1,16}$ = 1.96, p = 0.181; Condition by Agar interaction: $F_{1,16}$ = 1.96, p = 0.181), thus samples were pooled to measure the difference in richness by bacterial source.

There was a significant difference in richness among bacterial source (p < 0.001). Post-hoc analysis with Tukey's Honestly Significant Differences test (HSD) showed that water-only samples had significantly more species per sample (5.65 ± 0.55 SE) than the stressed anemones (2.67 ± 0.55 SE), normal anemones (3.10 ± 0.60 SE), or mucous samples (1.4 ± 0.40 SE), which were all similar to each other (**Figure 2**). No bacterial colonies were found growing on either set of controls during the experiment (n = 2 for each control type).

To characterize differences in bacterial community composition among treatments, we used non-metric multidimensional scaling (NMDS), a multivariate ordination technique, to plot the matrix of OTUs absent or present (coded as a 0 or 1) in each petri dish in two-dimensional space. This technique allowed us to quantify and visualize the relative similarity of the bacterial community among dishes, taking into consideration both the frequency and identity of the OTUs present (**Figure 3**). We used the resulting NMDS values assigned to each replicate to test the influence of culture conditions and agar salinity on the bacterial community from the water-only samples with a two-way ANOVA. There

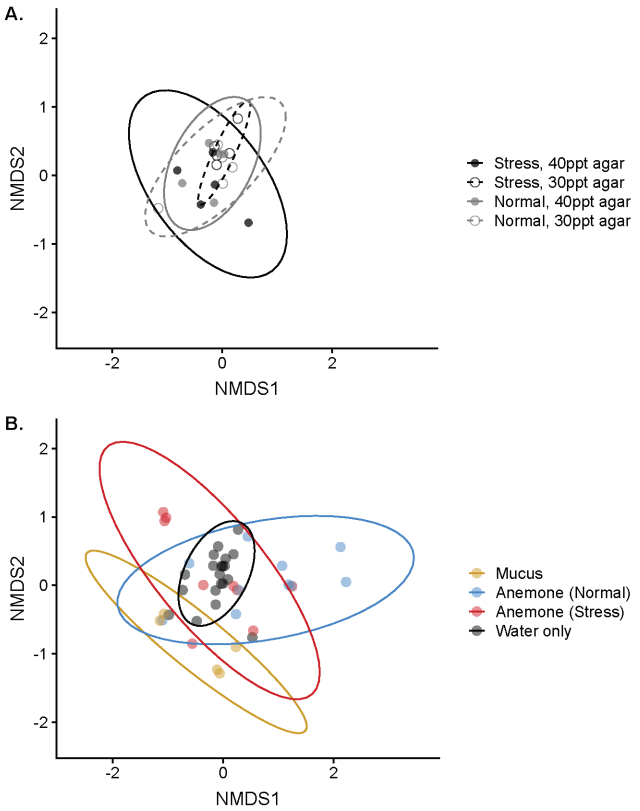

Figure 3. Non-metric multidimensional scaling plots of the bacterial community cultured on agar plates inoculated with fluid from experimental groups. Each point reflects the composition of the community on a single agar plate. Distance between points is correlated with dissimilarity among communities. (**A**) Variation among seawater samples that differed in culture conditions (point color) and the salinity of the agar (open vs. closed points) on which bacteria were plated. There was no significant difference in the average value of NMDS1 or NMDS2 due to either culture condition ($F_{1,16}$ = 0.30, 0.02; p = 0.595, 0.883), agar salinity ($F_{1,16}$ = 0.53, 1.91; p = 0.476, 0.186), or their interaction ($F_{1,16}$ = 0.60, 2.46; p = 0.449, 0.136). (**B**) Variation among communities by source (colors), including anemone-associated bacterial communities when anemones were grown under stressful or normal culture conditions, the mucus collected from stressed anemones, and water (samples combined across treatments). Groups differed significantly below the Bonferroni adjusted alpha (i.e., p < 0.025) along both NMDS1 ($F_{3,40}$ = 4.24, p = 0.011) and NMDS2 ($F_{3,40}$ = 1.31, p = 0.002). Tukey's HSD posthoc analysis showed that normal condition anemones differed significantly from all other groups along NMDS1, and mucus differed significantly from all other groups along NMDS2. The bacterial community associated with stressed anemones did not differ significantly from water only samples (p >> 0.025). Ellipses show 95% confidence intervals for each group.

were no significant differences among water samples along either NMDS graph axis (**Figure 3A**). Thus, we pooled all of the water samples for the next analysis comparing each set of NMDS values among bacterial source (mucus, normal anemones, stress anemones, and water) with a one-way ANOVA for each axis. Differences among groups were significant along both axes below the Bonferroni adjusted alpha of 0.025 (**Figure 3B**). Tukey's HSD post-hoc tests indicated that the microbiome of anemones grown in normal conditions differed significantly from the water-only samples,

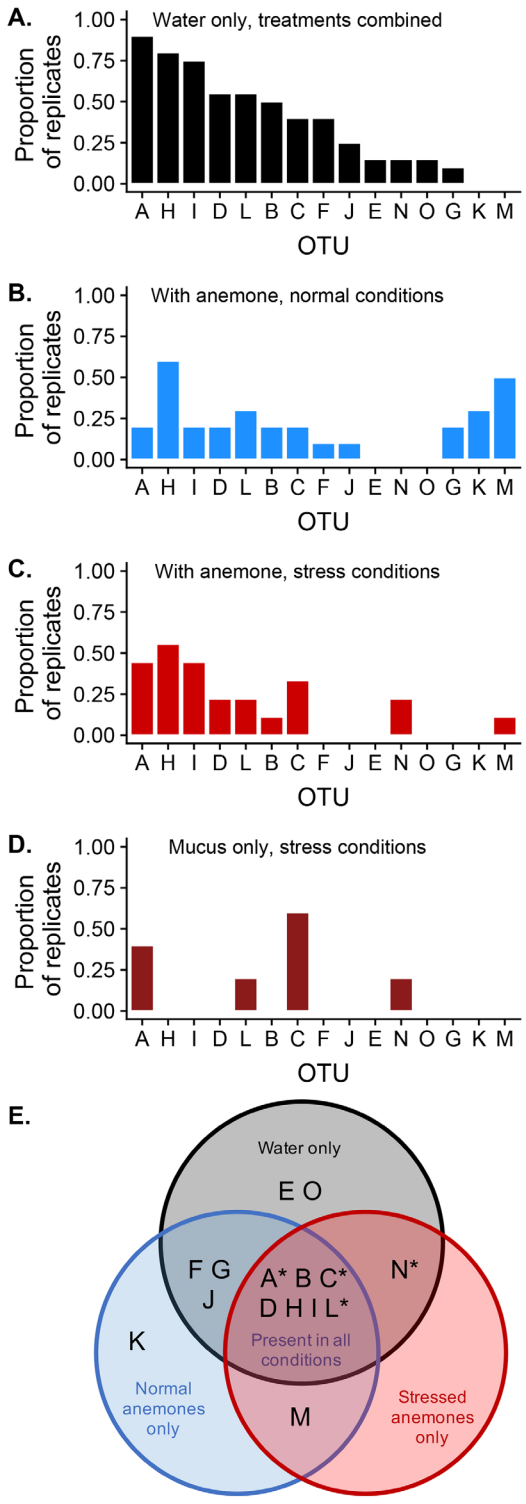

Figure 4. Frequency of occurrence of each OTU on culture plates inoculated with fluid from replicates of each condition. Bar order reflects rank order of commonness to rareness in water-only samples. Bar height indicates the proportion of replicates in which each OTU was found for each condition (panels **A-D**). Panels show the bacterial community composition in (**A**) all water only samples, combined across treatment, (**B**) anemone-associated samples grown in normal conditions, (**C**) anemone-associated samples grown in stressful conditions, and (**D**) excess mucus shed by anemones grown in stressful conditions. (**E**) Venn diagram of occurrence of putative OTUs in cultures grown from each treatment. Letters in overlapping regions indicate OTUs that were found at least once in petri dishes inoculated with fluid from conditions indicated in each circle. Letters in non-overlapping regions indicate OTUs that were unique to a single condition. Asterisks indicate OTUs that were present in mucous samples.

stressed anemones, and mucus (**Figure 3B**). Mucous samples differed significantly from all other treatments along NMDS2. The bacterial community from stressed anemones, however, was statistically similar to that found in the water samples (**Figure 3B**).

The patterns observed in the NMDS were also reflected in the distribution of common versus rare OTUs among treatments (**Figure 4**). The most common OTUs found across water-only plates (A, H, and I; **Figure 4A**) were also the most commonly found among replicates of the stressed anemones (**Figure 4C**). Many of the common OTUs found in water were sparsely found in normal anemone samples, except for H, which was commonly found with anemones also (**Figure 4B**). Likewise, two of the most common OTUs found with normal anemones, K and M, were only identified from anemone samples. OTU richness was low among the mucous samples but seemed to share the most similarities with the stressed anemones (**Figure 4D**). This includes higher representation of taxa A and C relative to normal anemones, as well as N, which was not found in normal anemones at all. The occurrence patterns within each treatment are summarized in a Venn diagram (**Figure 4E**) to demonstrate taxa which may contribute to distinct communities. Of particular interest are the two OTUs which only occurred with anemones (K and M), as well as the two OTUs which only occurred in water (E and O).

DISCUSSION

Emerging patterns from studies of sea anemones and corals suggest that the external environment plays a key role in shaping the richness and composition of associated microbial communities (4). The environment can influence the microbial community directly, by filtering the local species pool, and indirectly, by changing the physiology of the host species. Although the rudimentary techniques by which we have quantified bacterial diversity render our findings preliminary, our results are largely consistent with patterns observed by others.

We found that the composition of the bacterial community was different between unstressed anemones and the surrounding seawater, indicating that the host species can influence the bacterial community that assembles on its surface. Our detection of two OTUs associated with anemones that were not detected in the seawater samples suggested that the host can also cultivate taxa that are either rare or absent in the surrounding environment. Interestingly, when the anemones are stressed – as evidenced by the production of a mucous house – their microbiome becomes more similar to that of the surrounding seawater. This is consistent with

emerging evidence from corals that suggests that stressed hosts have a reduced ability to regulate their microbiomes (10). We did not find an increase in OTU richness or variance among replicate communities in stressed versus normal anemones, as has been reported for the sea anemone *Exaptasia pallida* (11). However, future studies using more sophisticated methods of species identification may uncover cryptic diversity within morphologically similar taxa. Finally, we found that the mucus produced by stressed anemones had a pattern of microbes different from either the seawater or the host anemones. Only four of the fifteen OTUs detected were found within the mucus. The low richness and unique composition of these communities supports the idea that the mucus may have some antimicrobial or otherwise selective properties, consistent with recent findings in corals (4) and other sea anemones (8). Future studies to examine the biochemical properties of anemone mucus and associated bacteria will likely provide fascinating results with insights for both pharmaceutical development and ecological understanding. The patterns we observe suggest that *D. lineata* shares many of the interesting features that have made corals and other sea anemones excellent models for studying the interaction of hosts and microbial communities with the external environment.

There are some important limitations to our findings. Primarily, our use of morphological features to determine bacterial OTUs meant that we likely underestimated the diversity present. In some cases, we may have also overestimated taxonomic diversity by misclassifying colonies of the same type as distinct due to plastic variation in colony morphology. Additionally, our techniques only revealed to us the subset of the bacterial community that is able to be cultured on agar plates. Future studies combining plating techniques with molecular analyses will allow us to estimate what fraction of the available bacteria were culturable and can help us better characterize the diversity present in these communities. However, the absence of bacteria in our negative controls suggests that our aseptic technique was successful. This means that the preserved samples from this and future studies have the potential to provide great insight when analyzed with genetic sequencing.

Understanding the mechanisms of microbial community assembly and succession on and within host species remains an area of active scientific inquiry. One area relevant to the biology of our sea anemone, *D. lineata*, is the role of microbes during species invasion. Our results show that *D. lineata* can both take on microbes from the surrounding seawater and modify the community present on tissues relative to the environment. Both of these qualities may be essential for a species that has been transferred to a new coastline. Continuing studies on the microbial communities of this species across its native and invaded range will allow us to learn much more about the interaction among hosts, microbes, and the external environment.

METHODS

Optimization of stressful versus normal culture conditions

Prior to the current study, a preliminary experiment was carried out to find an appropriate set of conditions to label as "stressful" to the anemones. Anemones were exposed to one of a factorial combination of cool and warm temperatures (10° versus 30°C) and water salinity levels (20, 30, 40 ppt) for two weeks. All anemones survived in all combinations, but the high salinity (40 ppt) and high temperature treatment (30°C) stimulated the most reliable production of mucous houses, thus we deemed these conditions stressful. Anemones grown at 10°C and an ordinary seawater salinity (30 ppt) behaved normally and showed no signs of stress during the experiment; thus, these conditions were deemed normal.

Sample collection

In June 2018, many *D. lineata* individuals were collected from a floating dock at King's Creek Marina in Cape Charles, Virginia. Anemones were transferred on ice in vials of natural seawater from the same site to the University of Alabama at Birmingham. Anemones were moved into vials of sterilized natural seawater collected from the same site and kept at 10°C until the start of the experiment. Under these conditions anemones can survive for long periods without food, which allowed us to avoid the potentially confounding effects of adding microbes associated with food.

Environmental influence on anemone microbiome

We designed an experiment to characterize the bacterial community associated with sea anemones grown in stressful and normal conditions (**Figure 1**). Natural, unsterilized seawater collected from the VIMS Eastern Shore Laboratory in Wachapreague, Virginia was used as a culture medium. Due to natural evaporation, the initial salinity was 40 ppt, which was used for the stress treatment. An aliquot of 40 ppt seawater was diluted with sterile deionized water to 30 ppt for the normal treatment (**Figure 1A**). Salinity was confirmed with a refractometer using aseptic technique at each step to avoid introducing new bacteria to either solution. For each condition, 3 mL of seawater was aliquoted into each of 10 wells of a 12-well tissue culture plate (Thermo Fisher Scientific, Waltham, Massachusetts). Twenty similarly sized *D. lineata* individuals were chosen from among the field-collected samples. The underlying genetic relatedness of the individuals was not known. One anemone was randomly assigned to each water-filled well. Plates were then placed in growth chambers (Percival Scientific, Perry, Iowa) with a 12:12 hour light cycle at either 30°C (stressed) or 10°C (normal) (**Figure 1B**). After seven days, each anemone was carefully transferred into 1.5 mL of sterilized seawater in a microcentrifuge tube using sterilized forceps. Nine out of ten stressed anemones produced mucous houses, while normal condition anemones did not. Each mucous house was carefully separated from its anemone and transferred to a separate tube of sterile seawater. All tubes were vortexed

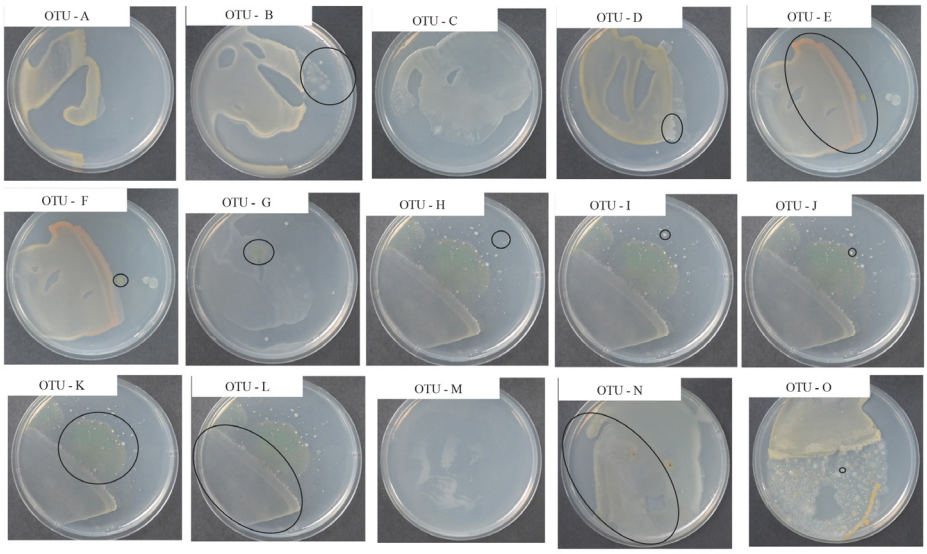

Figure 5. OTU identification guide. These images, along with a written description, were used as a reference to standardize the identification of OTUs across experimental images.

vigorously for 30 seconds to transfer bacteria to the sterile seawater. 1 mL of water from each tube was then spread onto an independent agar-filled petri dish (R-2A media prepared with 30 ppt seawater; Sigma-Aldrich, St. Louis, Missouri) (n = 10, 10, 9 for stressed anemones, normal anemones, and mucous houses, respectively; **Figure 1C**).

Environmental influence on seawater microbiome

Five wells of natural seawater for each culture condition were prepared as above and incubated in the same growth chambers simultaneously with the anemone inhabited plates (**Figure 1B**). After seven days, the water samples were plated. To ensure that the salinity of the agar plate did not inadvertently act as a filter on the bacteria that we observed, we plated one milliliter of water from each condition on R-2A media prepared with both 30ppt seawater and 40 ppt seawater (n = 5 for each combination of treatment by agar type; **Figure 1D**). We compared the influence of culture condition and agar on the OTU richness in water-only samples with a two-way ANOVA.

Controlling for contamination

To ensure that our results were not skewed by bacteria from any source except the samples, we prepared two replicates each of two controls. The first tested for contamination in the agar and consisted of two agar plates which remained closed after pouring and for the duration of the experiment. The second tested for contamination from the air during plate handling and consisted of two agar plates which were left uncovered on the lab bench for 30 seconds, which was much longer than any experimental plate was open during inoculation.

Growing and identifying bacterial colonies

All inoculated agar plates and controls were incubated at 25°C for seven days before photographing the bacterial growth. The images were used, without knowledge of which plate belonged to which treatment, to develop an identification guide of morphologically unique colony types, which were treated as OTUs. Fifteen such OTUs were identified, each assigned a letter A through O (**Figure 5**).

Using the OTU identification guide (**Figure 5**), the photograph of each plate was scored for the presence or absence of each putative taxa. In some cases, colonies had grown together on the plate which prevented us from quantifying the number of colony-forming units as a measure of abundance in each sample. Thus, only the presence and absence patterns were analyzed for this study.

Data analysis

Similarities among the bacterial communities cultured from each treatment were compared in four different ways. First, we compared the OTU richness based on bacterial source (i.e., water, stressed anemone, normal anemone, or mucus) with a one-way ANOVA.

Next, we used NMDS implemented in the VEGAN package of the statistical software R (v 3.5.1) to compare the similarity of the bacterial community among treatment conditions (12, 13). NMDS is a commonly used multivariate ordination technique that allowed us to reduce the complexity of the data for subsequent analysis. This technique was implemented with the metaMDS function in R. Briefly, the major axes of variation are found by first summing the number of differences in the presence or absence of OTUs between each possible pair of samples. Then, the rank order of differences between pairs of samples was used to position each sample on a set of X and Y axes such that more similar samples are plotted nearer to each other. This technique reduces the OTU data for each dish into a single point described by an X and Y value called NMDS1 and NMDS2. These values do not have a biological meaning, per se, but reflect the relative similarity or difference of the species composition between samples.

We used the NMDS values as the response variables in ANOVAs to test two null hypotheses. First, there was no

difference in bacterial community composition among water treatments due to culture condition or agar type, and second, there was no difference in bacterial community composition among the experimental conditions, i.e., found in mucus, with normal anemones, with stressed anemones, and in water samples (pooled across treatments). Because we used some of the same data for both null hypothesis tests, we used a Bonferroni correction to set a more conservative threshold for rejecting the null hypotheses (alpha = 0.025 instead of the conventional 0.05) to reduce the risk of type I error. Where a significant difference was found among groups using ANOVA, we used a Tukey's HSD post-hoc test to determine the pairwise pattern of significant differences among groups.

We then examined the distribution of OTUs within and among treatments by calculating and plotting the proportion of replicates in each condition where each OTU was present. This allowed us to visually compare patterns of commonness and rareness of OTUs among treatments.

Finally, we examined patterns of unique and non-unique OTU occurrences among treatments with a Venn diagram.

ACKNOWLEDGEMENTS

We thank Edward Smith from the Virginia Institute of Marine Science Eastern Shore Lab for field help and logistics, the staff at King's Creek Marina for access to their docks, and Dr. Jeff Morris at UAB for help with bacterial culturing. This project was funded in part by start-up funds from the University of Alabama at Birmingham to Dr. Stacy Krueger-Hadfield and the Office of Postdoctoral Education Career Enhancement award to Dr. Will Ryan. We would also like to thank the four anonymous reviewers whose comments improved this manuscript.

Received: May 19, 2020
Accepted: December 29, 2020
Published: February 15, 2021

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