Progress Report for NOAA Project #NA14NOS4820090 July 30th 2015
Title: Evaluating the mechanisms that drive nutrient-induced coral disease and bleaching

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**Project summary:**

We previously presented the first experimental evidence that chronic nutrient exposure leads to increases in both disease and bleaching in scleractinian corals. Despite those findings, it remained unclear whether excess nitrogen (N), phosphorus (P), or the combination (N+P) are the primary drivers of this observed increased in disease and bleaching. Furthermore, the mechanism that promotes nutrient induced disease and bleaching had yet to be elucidated. Thus this NOAA funded project #NA14NOS4820090 aimed to study how nutrient enrichment with N, P, or N+P alters coral physiology and/or the microbial communities associated with corals.

1.0 Overall Project Findings

With the NOAA grant funds we conducted a series of experiments that have now provided critically important data on the role of land-based sources of pollution in coral disease and bleaching. Importantly, during this experiment in the summer of 2014, a major thermal stress event occurred at our study site in the Florida Keys. This high temperature event provided us an interesting, albeit complicating, factor to consider when evaluating the threats to corals from inorganic nutrient inputs. Based on the gathered data we have arrived at several major conclusions about the roles of land-based sources of pollution through the lens of thermal stress.

1. Dark spot disease is induced by high thermal stress as well as cold stress.
2. The exposure to N alone, P alone, or N+P can exacerbate the effects of thermal stress on disease prevalence.
3. Elevated nutrients may prolong disease events by preventing recovery after thermal stress.
4. Application of N alone increases the photo-physiology of the treated corals, while the combination of N+P reduces it.
5. Pre-exposure to N alone may slow down bleaching, but ultimately reduces resilience of corals to bleaching induced mortality.
6. The relative abundance of mucus associated *Symbiodinium* is not different in healthy versus dark spot disease afflicted corals.
7. Dark spot disease is associated with a single potential pathogen, an epsilon-proteobacteria in the *Campylobacterales* family.

## 2.0 Field Work Update

In July of 2014, we established our NOAA funded field experiment to evaluate whether excess nitrogen (N), phosphorus (P), or the combination (N+P) is the primary driver of the increased coral disease and bleaching we previously reported. We selected 20 resident *Siderastrea siderae* and 20 resident *Agarcia* spp. colonies; these were used as controls (n=5/species), or amended with either N (n=5/species), P (n=5/species), or N+P (n=5/species). This was done at similar depths (~ 5 m), along 30 m long transects with an East-West heading at our pilot field site in Pickles Reef. Transects were a minimum of 30 m apart, and did not have any endangered coral species within the vicinity. We then identified individual colonies of *Agaricia* spp. and *S. sideraea* along the transects for experimentation. Only corals >10 $cm^2$ (mean ± SEM 29.85 ± 5.97 $cm^2$; 18.0 ± 2.95 $cm^2$ for *Agaricia spp.*; 31.48 ± 7.04 $cm^2$ for *S. sideraea*) and in excellent health were chosen for the experiment. No differences in size classes were identified for either the *S. sideraea* (1-way ANOVA, P = 0.253) or the *Agaricia* spp. colonies (1-way ANOVA, P = 0.378) among the different treatments.

Nails were driven into the reef and flags with numbers were added to the nails to identify each colony and its treatment. Prior to the experiment (July 11$^{th}$), corals were photographed, visually annotated for health metrics, mucus was collected, and PAM measurements taken. Then based on random assignments, the corals were subjected to their individual enrichment treatments (no enrichment, nitrogen alone, phosphorus alone, and nitrogen and phosphorus). This was done by drilling eight 1.5 cm holes into 15 cm sections of PVC pipe wrapped in mesh. Each tube was filled with slow release fertilizer (Florikan Slow Release Phosphate, Nitrate, or both) and secured 10 cm from the respective target coral. The start date of the experimental nutrient enrichments was July 14$^{th}$ 2014. Corals were then re-photographed and mucus was sampled on July 21$^{st}$ 2014, August 16$^{th}$ 2014, October 2$^{nd}$ 2014, November 15$^{th}$ 2014, and January 14$^{th}$ 2015. Also two water samples were taken at mid depth along each transect for comparisons to the coral microbiomes at all sampling dates. To ensure consistent nutrient delivery, fertilizer was replaced monthly after corals had been sampled. All field work was concluded in January of 2015 due to the high rates of coral mortality driven by extensive bleaching at the site (see below).

We used 16S rRNA gene surveys to study the microbial changes in the coral mucus across the course of treatment, focusing especially on whether detectable microbial changes accompanied specific routes to mortality or tissue loss in corals. We focused on mucus communities because these are thought to provide a barrier against invasion by opportunistic pathogens, and can be sampled non-destructively from the same individuals over time. We deemed other methods, such as harvesting live coral tissue, too harmful and invasive to the coral for our goal of monitoring the coral microbiome over the long term.

Coral-associated bacteria and archaea were collected using sterile syringe removal of the coral surface mucus layer23 on SCUBA. A sterile syringe was used to first agitate the coral and then remove 10 ml of mucus using negative pressure. Once on the surface, the sample was placed in a sterile 15 ml conical tube, frozen on dry ice for the return trip, and kept frozen until processing. At a number of time points, 15 ml water sample controls were collected from >1 m above the reef and treated identically to mucus samples in downstream processing.

### *2.1 Field Work Challenges and Opportunities*

The experimental portion of this project was aimed to be ~ 12 months, but due to significant loss of the experimental animals the field work was terminated early. Unfortunately, due to the high seawater temperatures and massive bleaching experienced in the Florida Keys in

the summer and fall of 2014, many of the control and experimental *Agaricia spp.* died. This ultimately reduced our analytical power and most of the trends we identified were not statistically different as a result. Regardless, we have uncovered important insights on the role of different nutrients in coral bleaching and disease as well as coral resilience.

## 3.0 Field Work Data Analysis

We originally had planned a 1 year enrichment experiment. However due to the severe mortality event associated with the mass bleaching, we chose to end the experiment in January. Thus the following data are based on six months of enrichment.

### *3.1 Temperature & nutrients both increase the prevalence of disease*

A surprisingly finding of this study was that disease prevalence increased even in controls during the massive thermal stress event that occurred in the Florida Keys in the summer of 2014 (Fig 1a.). Disease prevalence in *S. sideraea* corals (Fig 1a,b) went from a 0% in July 2014 to ~80% of all the controls within 2 months. DSS was reduced in October to 40% in the controls and remained steady at this level until January when we ended the experiment. This was an unexpected finding. While DSS is known to vary over time (Borger 2004; Gochfeld et al 2006) it has been shown to generally peak in the winter months, not the summer ones. **Thus this experiment has shown that temperature stress in either direction can cause DSS in *S. sideraea*.**

Interestingly the prevalence of disease also remained above 50% from September to the conclusion of the experiment for all of the nutrient treatments suggesting that **exposure to elevated nitrogen and phosphorus may contribute to higher incidence** (as opposed to prevalence) **of the disease**, at least in the short term (Fig 1a,b). Lastly this may suggest that during such thermal events the presence of **elevated nutrients may prolong the subsequent disease by preventing recovery**. We aim to test this question in future experiments.

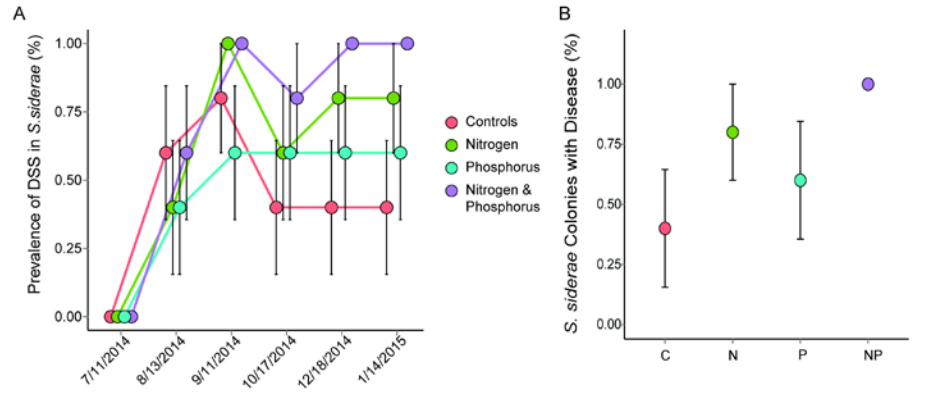


Figure 1. Effects of different nutrient enrichments on a) *Siderastraea* dark spot disease prevalence overtime, b) overall prevalence after 6 months treatment.

### *3.2 Nutrients lengthen and increase bleaching and mortality rates during thermal stress events*

During this high temperature event, high prevalence of bleaching and mortality occurred in our experimental *Agaricia* corals (Fig 2a,b). For example, while no corals showed signs of bleaching in July, 100% of the corals were bleached by August, independent of treatment.

However, by January control corals had recovered with only 20% of the corals being bleached. This was distinct from the experimental corals where bleaching prevalence remained at an average of 60% for all the treatments. This bleaching lead to high amounts of coral colony mortality with an average of 60% of the control corals dying and 100% of the nitrogen exposed

corals dying by January. **Together these data suggest that while prevalence of bleaching was not different across most of the bleaching event, recovery potential was different in the corals experimentally treated with nitrogen alone** (Fig. 2c). However, why nitrogen alone and not nitrogen and phosphorus showed this effect on recovery is not known, but using some of the physiology data we collected we can present hypotheses below.

*3.3 Nitrogen alone but not phosphorus or the combination alters symbiont photophysiology*

In addition to collecting coral health metrics, we conducted experiments to test how the treatments affected the *Symbiodinium*'s photophysiology using a technique called Pulse-

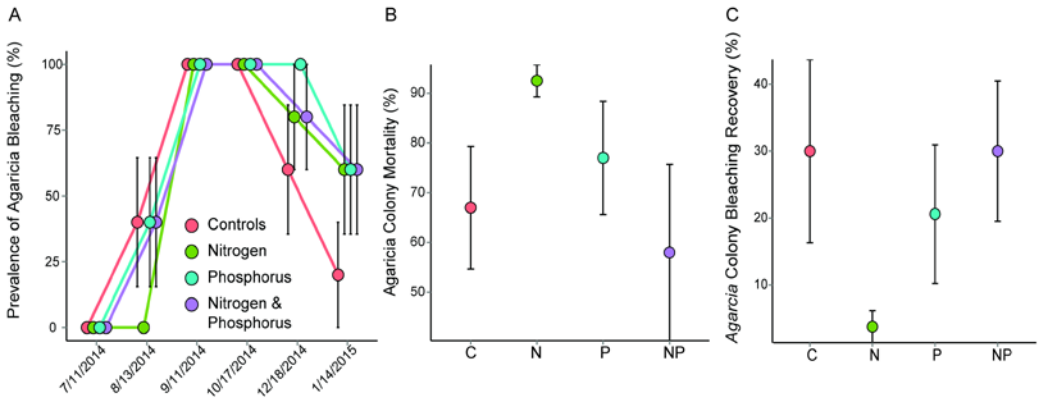


Figure 2. Effects of different nutrient enrichments on a) *Agaricia* bleaching overtime, b) mortality, and c) *Agaricia* bleaching recovery rates.

Amplitude-Modulation (PAM). PAM data collected in the first month of the treatment showed that application of nitrogen alone increased the photo-physiology of the treated corals but that the combination of N and P actually reduced it. These effects are fairly consistent with the literature where phosphorus has not been shown to have much of impact on the photobiology of corals, while nitrogen generally increases yield. Nitrogen in addition to phosphorus has been also shown to increase *Symbiondinium* density, but, counter-intuitively, this causes self-shading and therefore ultimately very little change in photosynthetic yield. One hypothesis is that nitrogen enrichment triggers bleaching by causing phosphorus starvation. This limitation shifts the symbionts requirement from N to P, and thus as *Symbiodinium* populations increase they consume all of the P resulting in symbionts that do not invest in adequate formation of phospholipid layers. The major physiological result is that this makes those symbiont lipid layers leak more reactive oxygen, triggering increased bleaching. This may explain why the NP corals recover slightly more; once those corals bleached, *Symbiodinium* populations start to recover and the additional P allows that, at least initially, they can build healthy phospholipid layers.

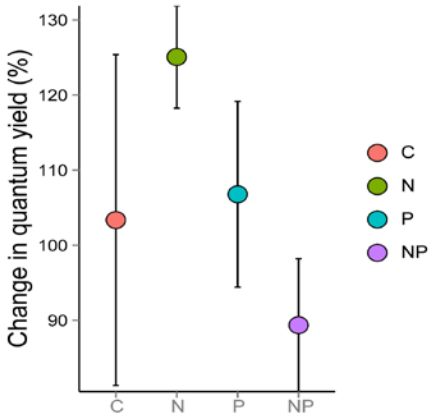


Figure 3. Effects of treatments on symbiont photo-physiology.

4.0 Nutrients and dark spot disease:

In an effort to determine the mechanism behind nutrient induced disease, we have processed all the *Siderastraea* samples for the generation of 16S amplicon and shotgun metagenomes. The metagenome library sequencing was completed in May 2015 (Table 1). In the original proposal, we had aimed to conduct multiple marker gene analysis to determine if the changes we had previously identified in nutrient exposed corals were the result of bacterial, viral, or eukaryotic pathogens. However, other work in our lab had determined that this approach was not sufficient for this study. Thus we switched from multiple marker gene analysis to just 16S rRNA (bacterial), ITS (*Symbiodinium* clade), and whole shotgun sequencing of the mucus samples (all taxa).

*4.1 Methods for whole-genome shotgun sequencing of coral mucus and reef seawater samples using multiplexed paired-end Illumina (HiSeq2000) technology.*

Initially, we were concerned that we would isolate mostly host DNA in the metagenomes, but we beta-tested this approach on other corals of the same species (collected in the same manner and same site) and found that the libraries contained remarkably few (<20-40%) sequences from the host. Thus we felt that this approach would give us a more comprehensive assessment of the overall potential pathogen pool. A major outcome of this work is the development of the following method that can be used by other scientists to generate microbial metagenomes from coral mucus.

Preliminary tests using our traditional DNA extraction approaches resulted in low DNA yields from the samples. Since metagenomics requires more DNA, we readjusted our extraction protocol accordingly. To increase the DNA concentrations, thawed coral mucus and seawater samples (10 ml) were concentrated using ultra-filtration centrifugal device (Centripep, Pall, 30 kDa cutoff) to a final volume of ca. 300 µl. Subsequently, DNA was isolated from sample concentrates using the Epicenter commercial DNA extraction kit (Illumina) following the manufacturer's instructions with some modifications. Purified DNA extracts were eluted in 20 µl of sterile water and kept at -20$^{o}$C. Purified DNA extracts were then quantified using the Qubit dsDNA assays (Invitrogen) and samples were diluted to a final DNA concentration of 1 ng/ul. Diluted genomic DNA extracts were fragmented and tagged for multiplexing with the Nextera XT DNA Sample Preparation Kit (Illumina) according to the manufacturer's instructions (version November 2014) with some modifications. Tagmented and indexed DNA were cleaned-up using magnetic beads for DNA purification (Ampure XP Beads, Agencourt). Purified Nextera libraries were quality checked using DNA fragment-size assays using the High Sensitivity DNA

BioAnalyzer system (Agilent 2100). Samples were pooled in equimolar concentrations. qPCR of the final pooled library will be carried out to achieve optimal cluster density on the HiSeq 2000 sequencer. Multiplexed DNA libraries will be sequenced using the Illumina HiSeq platform at the Center for Genome Research and Biotechnology facility at OSU in March of 2015. For this 1$^{st}$ round of sequencing, 88 barcoded DNA libraries were prepared from only the *Siderastraea* mucus samples (n = 80) and reef seawater samples (n= 8) collected on 4 dates: July 14$^{th}$, August 16$^{th}$, October 2$^{nd}$ and November 15$^{th}$. All the pooled samples were sequenced on a single lane on the HiSeq 2000 sequencer in May 2015. All samples were found to have yielded high numbers of reads with the exception of two samples (S7, S18) that will require resequencing. Using this method, we generated 88 total libraries that contained an average of ~5.2 million (range; 0.8 – 46 million reads) high quality 100 bp mean length sequence reads per sample (Table 1). Further these data are now freely available on the Mg-Rast server.

| Sample | Date | Treatment | # Reads | % of ≥ Q30 | Sample | Date | Treatment | # Reads | % of ≥ Q30 |
|---|---|---|---|---|---|---|---|---|---|
| S20 | 14-7 | C | 3,520,020 | 89.64 | S6 | 14-7 | NP | 9,889,976 | 88.58 |
| S3 | 14-7 | C | 3,960,020 | 89.62 | S14 | 14-7 | NP | 8,642,392 | 88.98 |
| S5 | 14-7 | C | 2,553,668 | 88.35 | S8 | 14-7 | NP | 7,815,494 | 87.34 |
| S9 | 14-7 | C | 9,678,784 | 88.51 | S1 | 14-7 | NP | 3,493,490 | 88.44 |
| S12 | 14-7 | C | 674,660 | 88.1 | S16 | 14-7 | NP | 474,154 | 89.25 |
| S5 | 14-9 | C | 4,473,072 | 88.32 | S16 | 14-9 | NP | 4,374,668 | 89.07 |
| S20 | 14-9 | C | 4,489,466 | 89.02 | S6 | 14-9 | NP | 5,791,162 | 88.77 |
| S12 | 14-9 | C | 6,473,708 | 89.45 | S14 | 14-9 | NP | 4,719,180 | 88.36 |
| S3 | 14-9 | C | 9,945,962 | 89 | S1 | 14-9 | NP | 2,615,442 | 88.31 |
| S9 | 14-9 | C | 2,351,646 | 88.68 | S8 | 14-9 | NP | 3,895,442 | 88.7 |
| S20 | 13-8 | C | 1,685,280 | 89.84 | S16 | 13-8 | NP | 1,507,922 | 89.74 |
| S5 | 13-8 | C | 949,170 | 89.47 | S8 | 13-8 | NP | 504,532 | 84.36 |
| S3 | 13-8 | C | 7,349,140 | 90.03 | S6 | 13-8 | NP | 2,317,904 | 89.54 |
| S12 | 13-8 | C | 3,678,414 | 89.04 | S1 | 13-8 | NP | 1,556,338 | 87.83 |
| S9 | 13-8 | C | 4,312,652 | 89.73 | S14 | 13-8 | NP | 1,511,494 | 88.29 |
| S3 | 17-10 | C | 3,441,300 | 90.17 | S6 | 17-10 | NP | 810,722 | 89.45 |
| S12 | 17-10 | C | 6,677,300 | 89.76 | S1 | 17-10 | NP | 551,818 | 88.89 |
| S20 | 17-10 | C | 2,861,290 | 90.02 | S16 | 17-10 | NP | 798,592 | 88.91 |
| S9 | 17-10 | C | 11,682,346 | 89.16 | S14 | 17-10 | NP | 2,526,566 | 89.15 |
| S5 | 17-10 | C | 836,360 | 89.38 | S8 | 17-10 | NP | 3,209,994 | 85.45 |
| S15 | 14-7 | N | 5,324,156 | 88.56 | S19 | 14-7 | P | 13,352,194 | 89.13 |
| S18 | 14-7 | N | 450,178 | 87.75 | S13 | 14-7 | P | 6,281,130 | 88.08 |
| S2 | 14-7 | N | 5,669,970 | 89.27 | S4 | 14-7 | P | 6,577,756 | 89.34 |
| S10 | 14-7 | N | 5,525,900 | 88.79 | S17 | 14-7 | P | 9,185,564 | 89.22 |
| S11 | 14-7 | N | 4,011,386 | 88.33 | S7 | 14-7 | P | 3,069,110 | 88.33 |
| S15 | 14-9 | N | 9,238,678 | 88.97 | S4 | 14-9 | P | 8,328,034 | 85.08 |
| S11 | 14-9 | N | 7,948,666 | 88.7 | S19 | 14-9 | P | 7,370,474 | 89.2 |
| S10 | 14-9 | N | 6,271,772 | 89.33 | S7 | 14-9 | P | 7,948,190 | 89.24 |
| S18 | 14-9 | N | 5,725,144 | 89.66 | S13 | 14-9 | P | 8,351,698 | 89.25 |
| S2 | 14-9 | N | 3,257,542 | 88.65 | S17 | 14-9 | P | 7,949,562 | 88.13 |
| S15 | 13-8 | N | 8,258,470 | 89.58 | S19 | 13-8 | P | 4,027,634 | 89.46 |
| S10 | 13-8 | N | 11,340,584 | 89.98 | S4 | 13-8 | P | 1,208,774 | 89.53 |
| S18 | 13-8 | N | 820 | 66.31 | S17 | 13-8 | P | 3,778,108 | 89.99 |
| S11 | 13-8 | N | 1,450,996 | 87.49 | S7 | 13-8 | P | 1,004 | 67.67 |
| S2 | 13-8 | N | 472,920 | 87.98 | S13 | 13-8 | P | 19,266,106 | 88.57 |
| S10 | 17-10 | N | 1,626,006 | 89.61 | S17 | 17-10 | P | 818,950 | 87.96 |
| S2 | 17-10 | N | 1,353,122 | 89.64 | S19 | 17-10 | P | 4,258,636 | 89.47 |
| S18 | 17-10 | N | 1,603,352 | 90.33 | S13 | 17-10 | P | 5,015,000 | 89.46 |
| S15 | 17-10 | N | 4,643,792 | 89.12 | S7 | 17-10 | P | 884,284 | 89.4 |
| S11 | 17-10 | N | 3,618,280 | 89.88 | S4 | 17-10 | P | 292,876 | 89.06 |
| HB | 14-7 | SW2 | 1,441,102 | 87.21 | HA | 14-7 | SW1 | 5,729,158 | 87.43 |
| HB | 14-9 | SW2 | 15,306,914 | 88.71 | HA | 14-9 | SW1 | 5,690,522 | 89.72 |
| HB | 17-10 | SW2 | 2,079,508 | 89.91 | HA | 13-8 | SW1 | 9,125,480 | 89.04 |
| | | | | | HA | 17-10 | SW1 | 1,270,922 | 88.7 |

Table 1.0. Basic metagenome high throughput sequencing results. C= control; N=nitrogen alone; P = phosophorus alone; NP = the combination treatment; SW= seawater.

*4.2 Metagenome sequence analysis*

We have been working with bioinformatian and microbiologist, Dr. Tom Sharpton, here at OSU, to assist us in these complicated and computer intensive analyses. Dr. Sharpton has developed a new method for shotgun metagenome library analysis called ShotMap (see previous report for details). Thus far we have helped Dr. Sharpton beta-test his program user options. We have started to use the novel Kraken bioinformatic tool (Wood and Salzberg, 2014) as a complementary approach to accurately and rapidly assign unassembled metagenomic reads. This approach relies on the decomposition of sequences into frequencies of short k-mers and makes use of machine learning techniques to train a classifier on a reference database. The taxonomic assignment of novel sequences is then predicted by applying a pre-trained model. Thus, short reads will be assigned to species only if they exactly match the k-mer frequencies of known genome models. Using this novel approach, we searched sample sequences against a custom made MiniKraken database that includes genomes from the Archaea, Bacteria and Virus RefSeq NCBI databases. This work is underway to evaluate, in-depth, the composition of the eukaryotic, prokaryotic and viral sequences. Ultimately these efforts will allow us to distinguish differences among the treatments and to answer the central question about the mechanism behind nutrient induced disease.

5.0 *Symbiodinium* analysis in exposed samples

Given the high prevalence of bleaching in our experimental samples, we are now attempting to evaluate the diversity and relative abundance of *Symbiodinium* types in these experimental corals. To do this we have begun to characterize the community using the *Symbiodinium* gene, ITS2. This gene is amplified using primers ITS-Dino-forward (5'-GTGAATTGCAGAACTCCGTG-3', Pochon et al. 2001) and its2rev2-reverse (5'-CCTCCGCTTACTTATATGCTT-3', Stat et al. 2009), as implemented in Green et al (2014, PeerJ) and Quigley et al. (2014, PLOS). To the 5' end of the *Symbiodinium* primers we have added Nextera adaptors (fwd: 5'-TCGTCGGCAGCGTC AGATGTGTATAAGAGACAG-3', rev: 5'-GTCTCGTGGGCTCGG AGATGTGTATAAGAGACAG-3') to allow for tag sequencing using the Illumina platform. For details see previous report.

*5.1 Symbiodinium communities are not different between healthy and DSS afflicted corals*

Thus far we have finished all the *Symbiodinium Siderastraea* samples and are in the process of sequencing the *Agaricia* samples. To analyze *Symbiodinium* amplicon data, a manually curated database has been constructed as follows. Well annotated *Symbiodinium* ITS2 sequences were downloaded from the ITS2 Database (Koetschan *et al.*, 2012), and clade/subclade taxonomy annotations were extracted and formatted for QIIME using custom shell scripts. MiSeq amplicon data were then processed in QIIME. Adaptor sequences were trimmed, paired-end reads stitched, OTUs clustered at 97% with UCLUST, and taxonomy assigned by BLAST against the custom database. These taxonomy assignments are used to assess the effects of our treatments on the differential relative abundance of the major *Symbiodinium* clades and subclades.

However, a major constraint of this experimental approach was that we could not take tissue samples across time. Thus, to beta-test that this approach conducted on mucus samples instead of tissue, was feasible, we used a previous collected dataset of *S. sideraea*.

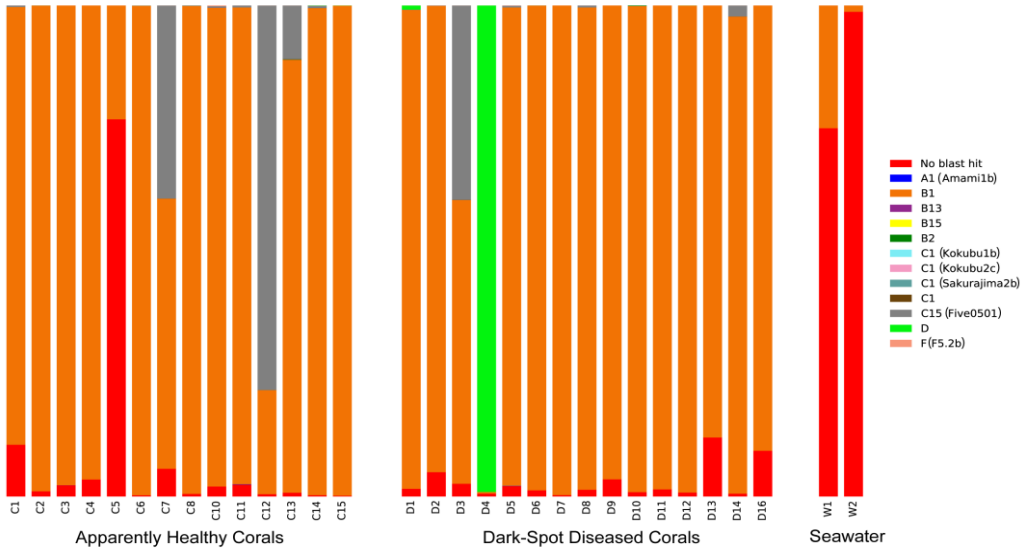


Figure 5. Analysis of Symbiodinium diversity in mucus samples from water, apparently healthy corals, and corals with visible signs of Dark Spot Disease. Each bar represents a single coral mucus or water sample and the different colors represent different symbiont types.

This old mucus dataset was from resident corals at our location that were apparently healthy or exhibited signs of dark spot syndrome. We paired these samples with water column samples collected above the corals to act as a control. Using the above described method, we found evidence that we could, at minimum, distinguish between the *Symbiodinium* community in coral hosts compared to those in the water column (Figure 5). Apparently healthy control corals exhibited a similar diversity of *Symbiodinium* clades compared to DSS but there was variation in the relative abundance or composition of the symbiont types. For example 1 DSS sample had a high relative abundance of D clade. However the *S. sideraea* corals were generally dominated by a single symbiont type, the B1 clade. Thus no major differences were seen in the symbiont types between healthy and DSS afflicted corals. We are still analyzing the samples from this NOAA funded project concerning the differential affects of the treatments on *Symbiodinium* community structure.

6.0 New sequencing efforts to investigate the origins of dark spot syndrome in corals

Dark Spot Syndrome (DSS) is a disease identified by darkened pigmentation of the coral tissue resulting in purple, black or brown lesions that can either be circular or elongate. A necrotizing disease, DSS can cause affected tissues to die at a rate of 4.0cm/month in *S. siderea* corals (Cervino, et al, 2001). Though it is often not obviously deleterious to whole coral colonies, it is a known marker for more aggressive diseases such as Black Band Disease and Yellow Band Disease (Cervino, et al 2001; Richardson 1998). Additionally, DSS affected corals are more likely to bleach than their healthy counterparts (Brandt & McManus, 2009). Previous work has shown that microbial communities associated with DSS lesions and healthy tissues have been shown to vary. In a study focusing on *S. intersepta* the microbes of healthy and diseased patches of coral were characterized. In DSS lesions, but absent in healthy tissue, four

types of pathogenic bacteria were identified (*Corynebacterium*, *Acinetobacter*, *Parvularculaceae* and *Oscillatoria*) along with the pathogenic fungi, *Rhytisma acerinum*. This work implicated that DSS in *S. intersepta* is caused not by a single pathogen but rather by a collection of taxonomically diverse microbes (Sweet, et al, 2011). More recently, and on the contrary, no difference was found between the bacterial communities in DSS lesions and those in healthy tissues of *S. siderea* (our target species), refuting the idea that DSS in *S. siderea* is linked to bacterial pathogenesis (Borger, 2005; Kellogg, 2014).

Thus a major aim of this project was to use high throughput sequencing to determine if any bacterium was associated with DSS and if nutrients influenced its abundance. To test this we used 16S and ITS amplicon as well as metagenome data to evaluate if corals exhibiting signs of dark spot had different microbial or *Symbiodinium* communities, respectively.

*6.1 Microbial community analysis using 16S rRNA amplicons*

For the bacterial community analysis of the individual nutrient treatments, we are still in the process of sequencing the longitudinal data. However, we have now conducted 16S analysis on a dataset of healthy and DSS afflicted *S. siderarea* under both ambient and N+P conditions. Using 25 healthy and 25 diseased samples and 16S amplicon data approach above, we found that although community structure as a whole is not different between the two health states, the relative abundance of a single bacterium was significantly more abundant in diseased corals.

To conduct this analysis the QIIME (v.1.8) software pipeline was used for quality control, selection of operational taxonomic units, and analyses of community diversity. Sequence libraries were demultiplexed, and sequences with quality scores less than a mean of 35 were removed. Error-correcting barcodes were used to detect and recover sequences whose barcode sequence had exactly 1 sequencing error. Barcode sequences with 2 or more errors were removed. Sequences were clustered into operational taxonomic units (OTUs), at a 97% 16S rRNA gene identity threshold using USEARCH 6.1.54430, and the subsampled open-reference OTU-picking protocol in QIIME v.1.8 31, using greengenes 13_8 as the reference32. This OTU-picking protocol clusters all reads, but assigns reference ids to OTUs in greengenes, which can be useful in comparisons across studies. Chimeric sequences were removed with UCHIME. OTUs represented in the overall analysis by only a single count (singletons) account for a large proportion of noisy reads. Because our emphasis was overall community trends (rather than exploration of the rare biosphere of corals), singleton sequences were removed. Representative sequences for each OTU were classified taxonomically according to the greengenes taxonomy version 13_8 using the RDP (Ribosomal Database Project) Classifier software v. 2.232,34. Sequence alignment and phylogenetic inference for the representative sequence of these OTU is described below in the context of beta-diversity analysis.

We also took additional steps to account for aspects of the dataset unique to host-associated samples. Because coral mucus can contain some amounts of sloughed tissue, we tested whether coral mitochondria were present in any mucus samples. Similarly, because *Symbiodinium* and other photosynthetic microbial eukaryotes frequently inhabit coral mucus, chloroplast sequences are frequently observed in microbial diversity surveys of corals. As our interest was primarily in cellular bacteria and archaea rather than organelles, coral mitochondrial sequences and 16S sequences classified as chloroplasts with at least 70% confidence by the RDP Classifier were removed prior to analysis.

Because we observed that many mitochondrial sequences were not efficiently identified by RDP, we also removed sequences with very high ($1\text{-}e^{-50}$) sequence similarity and at least 90%

sequence similarity to a reference coral mitochondrial sequence. These thresholds were selected with care to avoid indiscriminate removal of α-proteobacteria sharing evolutionary ancestry and 16S rRNA gene sequence similarity with mitochondria. The *e*-value for mitochondrial removal was selected by testing several e-values ($1\text{-}e^{-10}$,$1\text{-}e^{-30}$,$1\text{-}e^{-50}$,$1\text{-}e^{-100}$). For each *e*-value the best BLAST (Basic Local Alignment Search Tool [35]) similarity against NCBI's nr database was examined. We selected the highest (most lenient) *e*-value that removed mitochondria but not related bacteria. At a $1\text{-}e^{-50}$ BLAST *e*-value threshold, the best BLAST hit of all removed 16S rRNA sequences was coral mitochondria, and this threshold was therefore selected for the screen. OTU tables were summarized by taxonomy to the family level in QIIME 1.9. These data were imported into Phyloseq, and the formula DiseaseState. Treatment was used to fit a generalized linear model with DESeq2. Significance by treatment, disease state, and their interaction was determined using Wald tests in the DESeq2 package.

*6.2* Campylobacterales *are more abundant in dark spot afflicted corals than in healthy*

A single taxon within the order *Campylobacterales* (Class Epsilonproteobacteria) was significantly more abundant in the disease animals compared to the healthy. Further exposure to elevated nutrients increased the relative abundance of the taxa in both the healthy and in the diseased. These data suggest that nutrients mediate the growth of the taxa and contribute to disease. With these data, we now aim to specifically investigate the abundance of this taxa in the longitudinal data (both the metagenomes and 16S amplicons) created from the single enrichments.

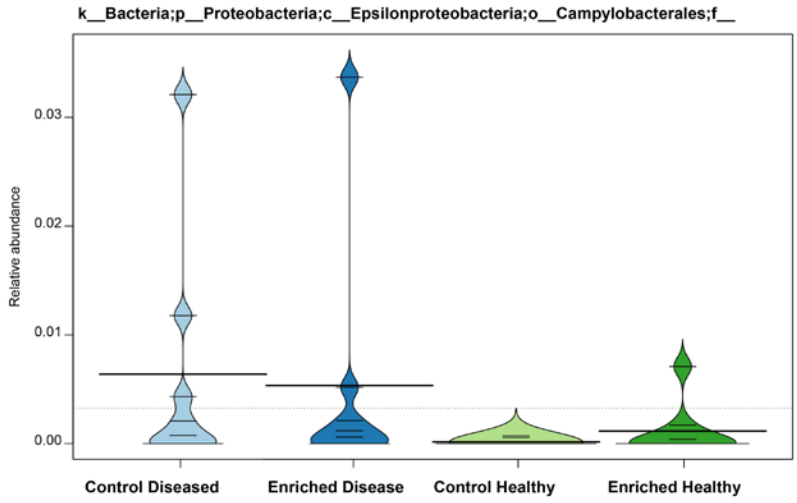


Fig. 6. Bean plots showing the median and range of relative abundance of a single significantly different bacterial taxon in diseased versus healthy animals. Diseased animals (blue) had more than 2x the abundance of an order of Epsilonproteobacteria, the *Campylobactererales*. While not significant, nutrient exposed (enriched) healthy corals tended to have more of this taxa than healthy corals in ambient nutrient conditions.

## 7.0 Future Work

A majority of the remaining work is the analysis and synthesis of the generated field and lab based data. We are currently running our metagenomes through the discussed pipeline and awaiting the sequence data from the longitudinal *Symbiodinium* ITS and bacterial 16S amplicons. We aim to have this work completed and prepared for submission and publication in a peer reviewed journal in the next year.

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