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Complex interactions between potentially pathogenic, opportunistic, and resident bacteria emerge during infection on a reef-building coral.

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Supplementary_Table3.csv Supplementary_Table4.csv	

Title: Complex interactions between potentially pathogenic, opportunistic, and resident bacteria emerge during infection on a reef-building coral.

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Running Title: Bacterial interactions during coral infection.

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Abstract

Increased bacterial diversity on diseased corals can obscure disease etiology and complicate our understanding of pathogenesis. To untangle microbes that may cause white band disease signs from microbes responding to disease, we inoculated healthy *Acropora cervicornis* corals with an infectious dose from visibly diseased corals. We sampled these dosed corals and healthy controls over time for sequencing of the bacterial 16S region. *Endozoicomonas* were associated with healthy fragments from 4/10 colonies, dominating microbiomes before dosing and decreasing over time only in corals that displayed disease signs, suggesting a role in disease resistance. We grouped disease-associated bacteria by when they increased in abundance (primary vs secondary) and whether they originated in the dose (colonizers) or the previously healthy corals (responders). We found that all primary responders increased in all dosed corals regardless of final disease state and are therefore un~~not~~ likely to cause disease signs. In contrast, primary colonizers in the families *Pasteurellaceae* and *Francisellaceae* increased solely in dosed corals that ultimately displayed disease signs, and may be infectious foreign bacteria involved in the development of disease signs. Moving away from a static comparison of diseased and healthy bacterial communities, we provide a framework to identify key players in other coral diseases.

Introduction

Marine invertebrates are home to some of the most widely studied and complex bacterial symbioses. The deep-sea hydrothermal vent tube worms *Riftia pachyptila* lack mouths or guts, instead acquiring nutrients from a specialized organ containing chemoautotrophic bacteria (Cavanaugh *et al.*, 1981). The bobtail squid, *Euprymna scolopes,* has also developed a specialized organ for its bacterial symbiont, *Vibrio fischeri,* allowing it to be bioluminescent (Nyholm & McFall-Ngai, 2004). As we learn more about these mutualistic relationships, we also better understand the continuum that lies between pathogens and beneficial symbionts. Theories posit that some beneficial bacteria may have originally been pathogens, evolving with the host to increase host fitness (Sachs *et al.*, 2011). Recently, the genome of a sulfur-oxidizing beneficial symbiont of deep-sea mussels was found to contain homologs of toxin-encoding virulence genes, complicating our understanding of pathogens and virulence (Sayavedra *et al.*, 2015).

The number of described marine diseases and their impacts have increased rapidly in recent years, contributing to the collapse of crucial marine ecosystems (Weil & Rogers, 2011, Burge *et al.*, 2014). This increase in epizootics is likely due in part to changes in marine bacterial-animal relationships as a result of anthropogenic inputs and the changing climate. Coastal marine ecosystems and the surrounding seawater are increasingly saturated with microbes profiting from rising temperatures (Tout *et al.*, 2015, Zaneveld *et al.*, 2016) and increased available nutrients due to both agricultural run-off (Garren & Azam, 2012) and a shift to algal-dominated ecosystems (Haas *et al.*, 2016). This increase in microbial abundance coupled with behavioral and gene-regulatory changes in previously benign bacteria have altered definitions of disease and symbiosis. In order to understand and ultimately control these new

epizootics, we need to examine how the bacterial communities associated with marine animals change, both as a cause of and in response to disease: shifting between beneficial, mutualistic, and pathogenic relationships.

White band disease (WBD) is an infectious disease currently decimating populations of the two species of Caribbean *Acropora* coral (*Acropora cervicornis* and *A. palmata*) (Randall & van Woesik, 2015). Acroporids are fast-growing reef-building corals that create habitats for numerous species of fish and invertebrates, including slower-growing species of corals (Gladfelter *et al.*, 1977, Tunnicliffe, 1983). WBD is characterized by a front of necrotic tissue (and sometimes a zone of bleached tissue), which proceeds rapidly from base to tip of the coral colony, leaving behind a band of white skeleton (Gladfelter, 1982). WBD can be transmitted through the water column and by the corallivorous snail *Coralliophila abbreviata* (Gignoux-Wolfsohn *et al.*, 2012). Multiple studies have confirmed that white band disease signs can be caused by the bacterial fraction of a disease slurry and arrested by the administration of antibiotics, suggesting a bacterial cause of the disease. (Kline & Vollmer, 2011, Sweet *et al.*, 2014). *Vibrio charchariae* has been shown to elicit WBD signs in *A. cervicornis* in Puerto Rico (Gil-Agudelo *et al.*, 2006), and a *Rickettsiales*-like organism, which may be compromising the host, has been associated with both apparently healthy *A. cervicornis* and *A. cervicornis* displaying disease signs (Peters *et al.*, 1983). How these and other bacteria contribute to the development of WBD signs and whether there is a single primary WBD pathogen across the Caribbean and through time is still unknown.

Previously, Gignoux-Wolfsohn and Vollmer (2015) used 16S gene sequences to find that WBD-associated bacterial communities were significantly different from those of healthy corals. In keeping with studies of other coral diseases, we found that the bacterial communities of corals

displaying disease signs were more diverse, with more consistently-associated OTUs than apparently-healthy corals (*e.g.,* Sunagawa *et al.*, 2009, Closek *et al.*, 2014, Roder *et al.*, 2014). The lack of consistent healthy-associated bacteria indicates that other factors may be shaping this microbiome. We also ~~found~~ showed that the site of collection influenced the microbial communities as much as the disease state of the coral ~~did~~. ~~;~~ T~~t~~he many disease-associated OTUs found ~~across~~ among all sites bec~~a~~ome many putative WBD pathogen(s). Bacterial diseases can be caused by a few cells of a single pathogen invading the host tissues (low infectious dose) e.g. (Zwart *et al.*, 2011), a consortium of pathogens that may be sufficient but not necessary to cause disease signs (Lemire *et al.*, 2015), or normally commensal bacteria reaching a threshold, which initiates a switch to pathogenic behavior (Rutherford & Bassler, 2012). Furthermore, commensal bacteria could become pathogenic due to an external environmental trigger (Lesser *et al.*, 2007). The uncertainty around which of these scenarios leads to the infectious white band disease-like signs complicates our ability to determine which of the identified "disease-associated" OTUs may be invading the host tissue and causing the disease signs and which may be responding to the necrosis, host immune response, or secondary metabolites produced by the pathogen(s). We exposed corals to an infectious dose of homogenized tissue from diseased corals and sampled corals ~~as they transitioned~~at three time points: ~~from~~ 1) apparent health prior to exposure, 2)~~to~~ apparent health ~~after~~ post exposure ~~to an infectious dose~~, ~~to the~~3) during the development of characteristic WBD signs. By using ~~corals from~~ multiple coral colonies, we were able to better identify resident microbes associated with each colony, and by performing this experiment in controlled tanks we removed the possibility of an environmental trigger of pathogenicity. We examined disease-associated OTUs for consistency across two sites in order to remove the ~~high~~ site variability we had previously found. This controlled infection experiment allowed us to

answer two main questions about the diseased coral microbiome: 1) Where do these disease-associated bacteria originate? And 2) when do they increase in abundance? We expected the final diseased-coral microbiome to be shaped by increased abundances of both bacteria originating in the dose (here referred to as colonizers) and bacteria that were found *a priori* on the corals (responders) increasing in abundance either before (primary) or after (secondary) development of disease signs.

Materials and Methods

Tank infection experiment

An infection experiment was set up in July 2014, using *Acropora cervicornis* from two sites (CK4 and CK14) 600m apart in Coral Cay, Bocas del Toro, Panama (*site*). At each site, corals to be inoculated were collected by taking twelve apparently healthy five cm fragments from five colonies (presumed to be distinct genotypes, at least 10 m apart) of *A. cervicornis* for a total of 10 colonies (*colony*) and corals to be made into inoculants were collected by taking three replicate five cm fragments from the disease interfaces (or equivalent location) of three colonies exhibiting signs of WBD and from three apparently healthy control colonies.

These fragments were brought to the Smithsonian Tropical Research Institute and the fragments to be inoculated were cable-tied to plastic louver. Ten fragments (one fragment from each colony) were ~~and~~ placed in each of 12 closed 50 L tanks (*tank*) with a powerhead See Fig. S1 for experimental design~~, 10 fragments (one fragment from each colony) per tank~~. Corals were sampled as they were placed in tanks (time one) in the following manner: two polyps from the middle of each fragment (this small sample was used so as not to stress the coral fragment) were removed using sterile forceps and placed in 200 µl of guanidine thiocyanate DNA Buffer (Fukami *et al.*, 2004). Forceps were flame sterilized in between corals. Throughout the

experiment, DI water was added to maintain salinity and volume and temperature was measured to ensure consistency across tanks.

To create ~~the~~ the 12 inoculants (three doses and three control inoculants from each site,) ~~,~~the t-hree replicate fragments from each colony were homogenized by shaking in a falcon tube with sterile glass beads and 15 mL filtered seawater until no tissue remained on the skeleton (Kline & Vollmer, 2011), and~~. Fragments from the same colony were~~ then pooled ~~to create three doses and three control inoculants from each site (*inoculant site*)~~. Two hundred µl of each inoculant was centrifuged and preserved in 500µl of DNA buffer.

Prior to inoculation, corals were lesioned using an airbrush and filtered seawater (Gignoux-Wolfsohn *et al.*, 2012). Six tanks were then inoculated with 30 mL of dose (the *dose* level of *inoculant*), three per site (*inoculant site*), ~~-~~and six tanks were inoculated with 30 mL of control inoculant (the *control* level of *inoculant*), three per site (*inoculant site*). Corals were then sampled at 10 hours post-inoculation as described above (time two). When dosed corals began to show disease signs (*i.e.* the white lesion grew to encircle the coral and form the characteristic white band of skeleton) beginning at 22 hours post-inoculation, they were sampled and removed from the experiment along with their corresponding control fragment (time three). Sampling continued in this manner until 60 hours post-inoculation when all remaining corals were sampled (See Fig. S1 for ~~experimental design and~~ sampling). The *final disease state* of a coral was determined based on whether or not that coral ultimately showed disease signs. For example, even though a sample collected at time two came from a healthy-looking coral, if that coral displayed disease signs at time three, the sample's final disease state was *diseased*. Forty-three out of 60 corals that were dosed ultimately displayed disease signs, ~~, and~~ T~~t~~wo of the 60 control corals died over the course of the experiment and were removed from subsequent analyses

(Table S1).

DNA was extracted from samples using the Agencourt DNAdvance bead extraction kit (Agencourt Bioscience Corporation, Beverly, MA, USA) with the addition of PEB buffer. A blank DNA extraction was performed with each round. The V6 hyper-variable region of the 16S gene was chosen as the target due to its short length and high sensitivity to species-level diversity (Youssef *et al.*, 2009, Barriuso *et al.*, 2011, Caporaso *et al.*, 2012). The V6 region was amplified with primers consisting of a region complementary to V6, a unique five base pair barcode, and the Illumina sequencing adapter (Gloor *et al.*, 2010):

V6-L [5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTnnnnnCWACGCGARG AACCTTACC3']

V6-R [5'CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnnACRACA CGAGCTGACGAC3']

A separate 40 µl PCR reaction was performed for each sample with a unique combination of primers: 5 µl each 4 mM primer, 8 µl standard Taq buffer (New England Biolabs, Ipswich, MA, USA), 0.8 µl dNTPs, 20 µl diH20, 0.5 µl Taq DNA polymerase (NEB) for the following cycle: 94°C for 2 m, with 28 cycles of: 94°C for 15 s, 55°C for 15 s, 72°C for 30 s, ending with 72°C for 1 m. A negative control and blank were amplified with each set of reactions. Concentrations of PCR products were quantified using the Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA) to determine the volume of each product to pool. The pooled PCR products were then amplified with the following Illumina primers:

OLJ139 [5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGA3']

OLJ140 [5'CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGC TGAAC3'] in a 40 µl reaction: 8 µl Phusion buffer (NEB) 0.8 µl dNTPs, 0.5 µl Phusion

HIfidelity Taq (NEB), 20.2 µl diH20, 0.5 µl DNA (previous PCR product), for the following cycle: 98°C for 2 m, 12 cycles of: 98°C for 1 m, 55°C for 1 m, 72°C for 1 m, and finally 72°C for 5 m. Final PCR products were cleaned using DNAmpure beads (Agencourt). Concentration and length were verified using the Agilent 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA) and sequenced using paired-end 150 base pair sequencing on an Illumina HiSeq2000.

Bioinformatics

Paired reads were overlapped using FLASH (Magoc & Salzberg, 2011). Sequences were then demultiplexed, quality filtered, and trimmed using a custom python script available at: https://github.com/sagw/Python_scripts/blob/master/SD1/SD1_demultiplex.py

Using Qiime 1.9.0, 97% OTUs were picked using the open reference OTU picking method and taxonomy assigned using BLAST against the July 2015 SILVA database (Quast *et al.*, 2013). OTUs that were identified as chloroplasts using BLAST were removed. Chimeras were detected and removed using UCHIME (Edgar *et al.*, 2011). Further details of bioinformatics can be found here:

https://github.com/sagw/Notebooks/tree/master/SD1_notebooks.

Statistical Analyses

OTU counts were normalized using the sizefactors method with arithmetic means in the R package DESeq2 (McMurdie & Holmes, 2014). The significance of the community level effects was tested using PERMANOVA of Bray-Curtis dissimilarities (adonis in package Vegan) (Jari Oksanen & O'Hara, 2013). Two PERMANOVAS were performed: one using the formula: "~ colony" for time one samples, and one using the formula: "~ final disease state + inoculant * site * time * inoculant site" for times two and three samples. Site was removed from the model because the main effect and interactions were not significant. ~~Diversity metrics~~Shannon

diversity and rarefied richness were calculated using Vegan (Jari Oksanen & O'Hara, 2013).

To evaluate changes in abundance of individual OTUs ~~across~~ among main effects and interactions with the addition of random effects, abundance data for each OTU were fit to quasipoisson mixed-effects generalized linear models (GLMMPQL in package MASS) (Venables & Ripley, 2003). GLMMs for time one samples used the fixed-effect formula: " ~ colony" and the random effect formula: "~1|tank". GLMMs for times two and three samples used the fixed effect formula: "~ final disease state + site * inoculant * time * inoculant site" and the random effect formula: "~1|tank/time". Significance of effects was then determined by type-III ANOVA using the Wald chi-square test (Anova in package Car) (Fox & Weisberg, 2011) and significantly different OTUs (p-value adjusted by false discovery rate <0.05) were determined for each main effect and interaction. OTUs were then grouped according to significance of GLMM terms and post-hoc calculated means and mean abundance of a subset of OTUs was plotted using ggplot2 (Wickham, 2009).

OTU Group Definitions

We identified colony-specific healthy residents as OTUs that differed significantly by *colony* at time one and by *final disease state* at times two and three, with a higher abundance in control than dosed diseased corals. The majority of these OTUs belonged to the genus *Endozoicomonas,* and so the mean of each OTU identified as *Endozoicomonas* was calculated for each colony and percent *Endozoicomonas* composition was calculated as a mean of the percent of the total microbiome for each sample belonging to a given colony.

~~Secondary OTUs differed significantly by *final disease state* and were more abundant in dosed diseased than control corals at time three but not time two. These OTUs were grouped by family, and means were calculated for dosed corals that showed disease signs at time three~~

~~separated by *site* and *inoculant site*.~~

We identified bacteria that are likely involved in the etiology of the disease (primary OTUs) as those OTUs that increased in abundance on corals that ultimately showed disease signs prior to the development of these signs. We assume that corals that were exposed to the infectious dose but did not display disease signs are resistant to the disease (*i.e.* decrease the pathogen load or prohibit infection) and may therefore not contain OTUs associated with the pathology of the disease within their microbiomes. We therefore focused on OTUs that were more abundant in dosed corals that ultimately displayed disease signs.

We identified OTUs as primary responders if they 1) were absent from the dose; 2) were present in time-one corals; 3) differed significantly by *final disease state* in time-two and three corals; 4) were more abundant in dosed diseased corals than controls; and 5) did not differ significantly by *colony*, *site*, or the interaction of *site* and *inoculant*.

We identified OTUs as primary colonizers if they: 1) were more abundant in the dose than the control inoculant; 2) differed significantly by *final disease state*; 3) were more abundant in dosed diseased corals than control corals at both times two and three; and 4) did not differ significantly by *colony*, *site*, and the interaction of *inoculant* and *inoculant site*.

Secondary OTUs differed significantly by *final disease state* and were more abundant in dosed diseased than control corals at time three but not time two. These OTUs were grouped by family, and means were calculated for dosed corals that showed disease signs at time three separated by *site* and *inoculant site*.

All sequences were deposited to the Sequence Read Archive under the bioproject ID PRJNA387312. Further specifics of analyses can be found here: https://github.com/sagw/R-scripts/tree/master/SD1.

Results and Discussion

We identified groups of OTUs that consistently changed in abundance, contributing to the characteristic diseased coral microbiome: a reduction in resident OTUs associated with certain coral colonies (colony-specific residents), an increase in other resident OTUs (primary responders), and colonization by foreign bacteria (primary colonizers). This method of identifying bacterial groups involved in the transition of a marine animal from health to visible disease signs can be applied to other underexplored marine diseases.

Community-level effects

Two hundred and seventy-five samples were sequenced yielding 65 413 553 overlapped reads, which resulted in 97 933 OTUs (97% similarity). The bacterial communities on dosed corals became dramatically more diverse as they developed disease signs in terms of Shannon diversity (from 2.13, SE 0.12 to 4.18, SE 0.19, ANOVA, $F_{1, 272}$=52.37, P<0.001) and rarefied richness (from 224.43 to 402.57, ANOVA, $F_{1,272}$ =27.95, P<0.001) (Table S2). This finding is consistent with other studies of coral disease-associated bacterial communities (*e.g.* (Croquer *et al.*, 2013, Sweet *et al.*, 2013, Gignoux-Wolfsohn & Vollmer, 2015, Meyer *et al.*, 2015)).

A large amount (18%) of the variation between bacterial communities of samples collected prior to dosing (at time one) was explained by the significant effect of colony (PERMANOVA, $F_{9,81}$=1.8, P=0.001, R^2=0.18). For samples collected after dosing (times two and three), the main effect of *final disease state*, and the interaction of *time~~point~~*, *inoculant*, and

inoculant site, significantly affected the coral-associated bacterial communities (Table 1).

Endozoicomonas are colony-specific residents of healthy corals

In contrast to studies of other species of coral, where *Endozoicomonas* dominate the microbiomes of all healthy individuals (Apprill *et al.*, 2013) (Bayer *et al.*, 2013) (Yang *et al.*, 2010, Klaus *et al.*, 2011, Jessen *et al.*, 2013, Roder *et al.*, 2015), reviewed in: (Neave *et al.*, 2016)), they were dominant residents of only four of the 10 colonies (marked as "High" in Fig. 1) of healthy *A. cervicornis,* comprising 139 of the 175 OTUs identified as colony-specific residents of healthy corals by GLMM (Table S3). *Endozoicomonas* have been shown to form species-specific associations, with different strains found in different species, but they have not previously been shown to vary so drastically ~~between~~ among colonies of the same species (Neave *et al.*, 2016). For these four colonies, *Endozoicomonas* may be beneficial, since they were less abundant in corals displaying signs of disease than in healthy controls, and were also less abundant in samples of these diseased corals collected at time two prior to visual disease signs (Fig. 2). These *Endozoicomonas* may only survive in healthy coral tissues; in other species of coral they have been identified in the endodermal tissues of the host coral (Bayer *et al.*, 2013). Alternatively, *Endozoicomonas* may be out-competed by the disease-associated bacteria as the coral contracts disease. Our results suggest that *Endozoicomonas* may help the coral fight off infection, as they were more abundant in corals that were exposed to the dose but remained healthy than in the healthy controls, which were never exposed to the dose (Fig. 2). A recent study found that removal of *Endozoicomonas* from the surface mucus layer of corals made corals more susceptible to bleaching and necrosis, highlighting the importance of *Endozoicomonas* in coral fitness and protecting against foreign bacteria (Glasl *et al.*, 2016). The observed colony-

specificity of *Endozoicomonas* residents could be due to both the host genetics and the environment. Colonies are likely genetically unique, and a high abundance of *Endozoicomonas* may be contributing to the disease resistance previously seen in certain genotypes of *A. cervicornis* (Vollmer & Kline, 2008, Libro & Vollmer, 2016). Colonies are also located in slightly different locations on the reef and *Endozoicomonas* abundance has been correlated to favorable environmental conditions (Roder *et al.*, 2015). Further investigation into the role *Endozoicomonas* may be playing in *A. cervicornis* health and disease resistance will be especially important given the recent finding that *Endozoicomonas* abundance within *Acropora muricatae* tissues decreases with increasing temperatures (Lee *et al.*, 2015).

Secondary OTUs are not consistent across site

The majority of the 1,906 identified secondary OTUs were neither responders (44 OTUs) nor colonizers (222 OTUs). These OTUs were, therefore, presumed to either have originated in the water or been at undetectable abundances when time one samples were taken. These OTUs appear to contribute the majority of the diversity found in bacterial communities of corals displaying disease signs, but in very low abundances. These low abundances likely~~,~~ contribute~~ing~~ to the difficulty in identifying important bacterial groups when comparing the bacterial communities of corals displaying disease signs to those of apparently healthy corals. Contrary to expectations, none of the secondary OTUs were consistent ~~across~~ among either site of origin of coral or dose. Rather, all 1 906 of these secondary OTUs were also significant for the interaction of "site," "inoculant," "inoculant site," and "time~~point~~" (Table S3, Table S4). These secondary OTUs are unlikely to be involved in development of disease signs, but are more likely attracted to the nutrient source of the dying coral. We only identified secondary OTUs (that were not unique to individual corals or tanks) that increased in abundance when the dose came from the

other site (*e.g.* CK4 corals inoculated with CK14 dose). In the dosed corals that developed disease at time three, 1 676 OTUs were more abundant in CK4 corals inoculated with CK14 dose and 230 OTUs were more abundant in CK14 corals inoculated with CK4 dose. *Francisellaceae* comprised the majority of these secondary OTUs on CK14 corals dosed with CK4 (55 OTUS), and had the second highest mean abundance after *Methylococcaceae*. For CK14 corals dosed with CK4, the most abundant family of secondary OTUs with the highest number of OTUs was *Campylobacteraceae* (358) (Fig. 3). ~~Since the~~The lack of consistency of secondary OTUs ~~were not more abundant on corals~~ across dose site and site indicates that~~,~~ they are unlikely to be playing a significant role in disease causation. Rather, this pattern suggests that there is ~~only~~ an additional~~a~~ secondary disturbance of the bacterial community when the disease-associated bacteria are not taken from surrounding corals.

Primary responders are potential opportunists

We classified bacteria that were already present in lower numbers on the healthy corals before dosing and responded to the dose by growing more abundant as primary responders. Contrary to our expectations and previous studies suggesting coral disease is caused by opportunistic pathogenesis of resident bacteria (Chow *et al.,* 2011; Lesser *et al.,* 2007), all 272 primary responders became more abundant after dosing in all dosed corals regardless of their final disease state (Fig. 4, Table S3). These OTUs, equally abundant in dosed corals that remained healthy and dosed corals that displayed disease signs, are unlikely to be the sole cause of the disease.

Primary responders in the phylum *Bacteroidetes*, which includes families *Flavobacteriaceae* (26 OTUs), *Cryomorphaceae* (22 OTUs), and *Saprospiraceae* (20 OTUs),

appeared to respond to both the disease dose and the general stress of the tank environment by increasing in all corals (including controls) at time three (Fig. 4). We previously found many OTUs belonging to *Flavobacteriales* (which includes both *Flavobacteriaceae* and *Cryomorphaceae*) consistently associated with WBD-infected corals (Gignoux-Wolfsohn and Vollmer 2015). *Flavobacteriaceae* have been associated with many coral diseases across oceans (*e.g.* (Apprill *et al.,* 2013; Frias-Lopez *et al.,* 2002; Ng *et al.,* 2015; Roder *et al.,* 2014b)), cause disease in fish (Starliper 2011), and are part of some healthy marine microbiomes (Apprill *et al.,* 2014). *Flavobacteriaceae* were recently found to be enriched on algae-dominated reefs, which contain more readily accessible dissolved organic carbon (Haas *et al.,* 2016). Their high abundance in the closed aquaria, which likely grew increasingly nutrient-rich as corals died, is consistent with their functioning as copiotrophs; they may be blooming as they consume the dying coral or the secondary metabolites of other members of the diseased bacterial community. We previously identified strains of *Saprospiraceae* associated with both diseased and healthy corals (Gignoux-Wolfsohn and Vollmer 2015). Members of this family include commonly found marine bacteria involved in the breakdown of complex carbon molecules, consistent with their possible response to a dying or stressed coral (Krieg *et al.,* 2011).

Rather than continuing to increase over time, primary responders belonging to the family *Alteromonadaceae* (24 OTUs) were most abundant at time two, before any corals displayed disease signs (Fig. 4). These OTUs may grow as an initial response to the introduction of foreign microbes, possibly as defensive symbionts of the host coral. *Alteromonadaceae* have been previously associated with healthy coral larvae (Ceh *et al.,* 2013) and healthy adult corals (Cardenas *et al.,* 2012), suggesting they can be beneficial symbionts. They are also, however, more abundant in corals infected with multiple diseases (Frias-Lopez *et al.,* 2002; Gignoux-

Wolfsohn and Vollmer 2015; Roder *et al.,* 2014a; Roder *et al.,* 2014b; Sunagawa *et al.,* 2009), consistent with a role as defensive symbionts. *Rhodobacteraceae*, the bacterial family most widely associated with coral diseases (summarized in: (Mouchka *et al.,* 2010) see also:(Cardenas *et al.,* 2012; Gignoux-Wolfsohn and Vollmer 2015; Ng *et al.,* 2015; Roder *et al.,* 2014a; Roder *et al.,* 2014b)), contained many primary responders (18 OTUs) responding to the dose not the final disease state of the coral (Fig. 4). *Rhodobacteraceae* seem to be important players in health and disease ~~across~~ for multiple coral species (Glasl *et al.,* 2016; Mouchka *et al.,* 2010), possibly as opportunists or as defensive symbionts, helping the host to fight off infection by foreign bacteria.

Primary colonizers are likely putative primary pathogens

We identified primary colonizers as 265 OTUs that originated in the disease dose and preferentially colonized corals prior to the development of disease signs, likely after chemotaxing through the water column towards the host coral and attaching to its surface (Fig. 5, Table S3). The coral pathogen *Vibrio corallilyticus* uses the coral metabolite dimethylsulfoniopropionate to locate potential hosts (Garren *et al.,* 2014); it is possible that the pathogen(s) may use a similar method of host location. It is interesting, therefore, that we did not only identify one species of bacteria that originated in the dose and colonized corals prior to disease signs, but rather many sometimes distantly related OTUs. This result likely explains the difficulty in identifying primary pathogens of coral diseases and indicates that there may not be a single primary pathogen, but a consortium of bacteria that cause disease signs. Evidence that quorum sensing is important in contraction of WBD provides a possible method for inter-species communication and infection by a consortium (Certner and Vollmer 2015). The previously suggested WBD pathogens, *Vibrionaceae* (one OTU), and *Rickettsiaceae* (5 OTUs), were not more abundant in dosed corals that displayed disease signs than those that remained healthy,

making them unlikely primary pathogens in this experiment (Fig. 5).

Interestingly, taxonomy did not always dictate where and when OTUs were found. Many primary colonizers and primary responders were identified as belonging to the families *Flavobacteriaceae* and *Alteromonadaceae*. The 22 *Flavobacteriaceae* identified as colonizers again appear to be acting as copiotrophs, and may have been abundant in the dose because they were primary responders on the corals used to create the dose. In contrast, the 22 *Alteromonadaceae* identified as primary colonizers followed a different pattern from *the* Alteromonadaceae OTUs in the primary responders group. Instead, their pattern of abundance was similar to primary colonizer OTUs belonging to other families including *Campylobacteraceae* (25 OTUs), *Francisellaceae* (38 OTUs), and *Pasteurellaceae* (26 OTUs)—only colonizing corals prior to the development of disease signs, and proliferating as the disease progressed (Fig. 5).

The absence of many groups of primary colonizers from corals that were dosed but did not display disease signs indicates that these OTUs are likely directly involved in the development of WBD signs (Fig. 5). Members of the *Campylobacteraceae* family have been associated with multiple coral diseases including WBD (*e.g.* Gignoux-Wolfsohn and Vollmer 2015; Roder *et al.,* 2014a; Sunagawa *et al.,* 2009; Sweet and Bythell 2012; Sweet *et al.,* 2013). In other systems, *Campylobacteraceae* are known to be both commensal and zoonotic pathogens (Lee and Newell 2006; Stoddard *et al.,* 2005). In contrast, *Francisellaceae* have not been previously associated with coral disease, but are common marine bacteria (Duodu *et al.,* 2012), which can be intracellular pathogens of both Atlantic cod (Wangen *et al.,* 2012) and humans (Sjostedt 2006) and are also endosymbionts of ciliates (Schrallhammer *et al.,* 2011).

Primary colonizers in the family *Pasteurellaceae* exhibited a pattern of colonization

consistent with a strong involvement in disease: these OTUs were very abundant in the dose and preferentially colonized dosed corals before they showed disease signs with a more dramatic increase in abundance than any other family (Fig. 5). *Pasteurellaceae* have not been previously associated with coral disease, but they are common pathogens of many other animals including humans (Frey and Kuhnert 2002; Johnson and Rumans 1977) and were recently found to be enriched on reefs with high algal cover (Haas *et al.,* 2016). One possible explanation for our identification of *Pasteurellaceae*, and not *V. charchariae* behaving like a primary WBD pathogen, is that *Pasteurellaceae* may be an emerging pathogen of Panamanian corals that also causes WBD-like signs. The increasingly algae-dominated Panamanian reefs may promote new coral pathogens that cause macroscopic signs similar to canonical WBD.

While we identified some consistent actors in the diseased coral microbiome, we did not explain the majority of variation between samples, indicating there are other factors not examined in this study that shape the coral microbiome. This study used corals displaying disease signs consistent with WBD from Panama; whether the patterns described here apply to all corals displaying WBD-like signs across the Caribbean is unknown. We were limited by the length of the region sequenced and the available databases—as technology and resources improve, bacterial taxonomy will be better resolved.

Conclusions

The diseased coral microbiome is dependent on the pre-existing healthy microbiome, the disease history of the infected coral, the origin of the disease, and the timing of disease progression. Our approach allowed us to separate bacteria based on origin and timing of increased abundance, providing more information than previous culture-independent studies about what bacteria are likely contributing to disease. Our finding that *Endozoicomonas* are only

associated with health on certain coral colonies may explain the variation in responses of individual corals to disease. The discovery that primary responders, likely opportunists, increase in dosed corals regardless of final disease state negates hypotheses that white band disease on *A. cervicornis* is ~~coral diseases are~~not caused solely by opportunists. We identified primary colonizers originating in the infectious dose and were able to closely track their changes in abundance as corals developed disease signs, identifying *Campylobacteraceae*, *Francisellaceae*, and *Pasteurellaceae* as the most likely primary pathogens. Our results underscore the importance of incorporating time into future studies of marine diseases and the need to observe the behavior of individual bacterial strains rather than summarizing changes in communities only by higher-level taxonomy. Our approach can be applied to other marine diseases that do not fit into a one-pathogen one-disease framework, providing a more holistic understanding of disease and allowing for the shifting definitions of pathogens within our changing marine climate.

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Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Fig. 1. *Endozoicomonas* are colony-specific resident bacteria of healthy corals. a) Mean abundance of each resident OTU within each colony at time one, black bars denote standard error. Colonies with greater than 40% of their total microbiome consisting of *Endozoicomonas*

Comment [SGW1]: Check that this is true and its not sd or something else?

at time one are labeled "High." b) Percent of total microbiome for each colony at time one that is identified as *Endozoicomonas* or other taxa.

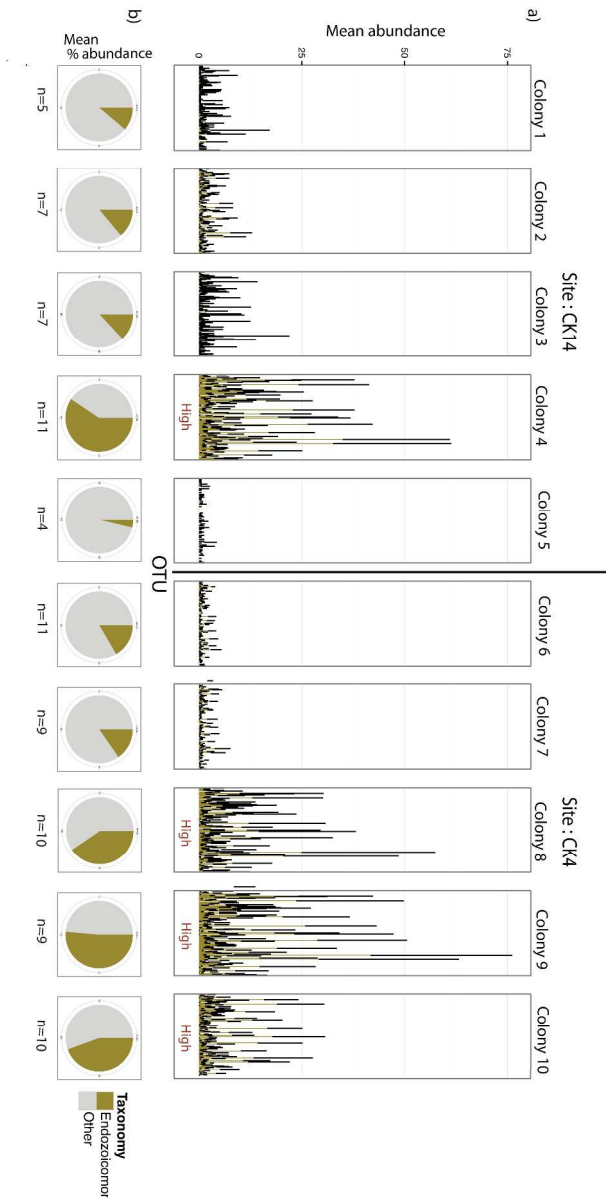
Fig. 2. Abundance of resident *Endozoicomonas* in colonies with greater than 40% of their total microbiome consisting of *Endozoicomonas* at time one (high) in dosed corals times two and three. Y-axis is the difference between dosed corals and control corals at each time point; a negative value denotes a lower abundance in dosed corals than controls, and a positive value denotes a higher abundance in dosed corals than controls. Means were calculated for corals exhibiting different final disease states (diseased or healthy) and then control means were subtracted.

Fig. 3. Mean abundance of secondary OTUs belonging to selected families on dosed corals that became diseased at time three. Dosed corals are separated by the site of origin of the dose and the site of origin of the corals. OTUs are grouped by family, and the number of OTUs in each group is noted on the top of the mean abundance bar.

Fig. 4. Mean abundance of primary responders belonging to selected families across time. OTUs are grouped by family, and the size of the points denotes how many OTUs belonged to that family.

Fig. 5. Mean abundance of primary colonizers belonging to selected families across time. OTUs are grouped by family, and the size of the points denotes how many OTUs belonged to the

specified family. Inset is the mean abundance for OTUs in that family in the inoculants (dose and control). Arrows signify time of inoculation. Error bars denote standard error.



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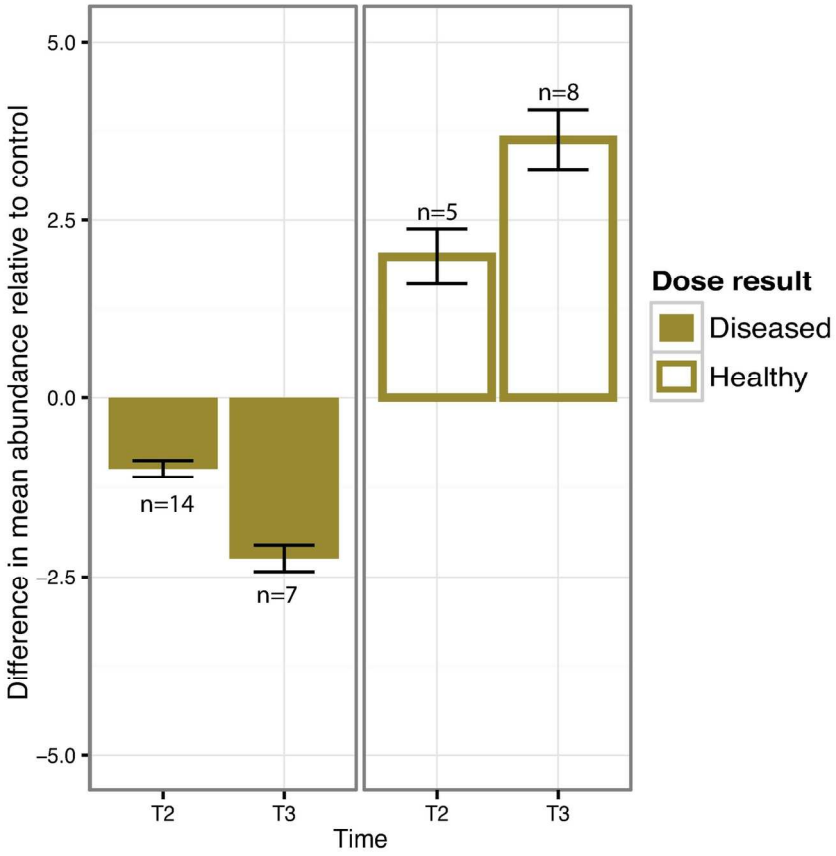

Fig. 2. Abundance of resident Endozoicomonas in colonies with greater than 40% of their total microbiome consisting of Endozoicomonas at time one (high) in dosed corals times two and three. Y-axis is the difference between dosed corals and control corals at each time point; a negative value denotes a lower abundance in dosed corals than controls, and a positive value denotes a higher abundance in dosed corals than controls. Means were calculated for corals exhibiting different final disease states (diseased or healthy) and then control means were subtracted.

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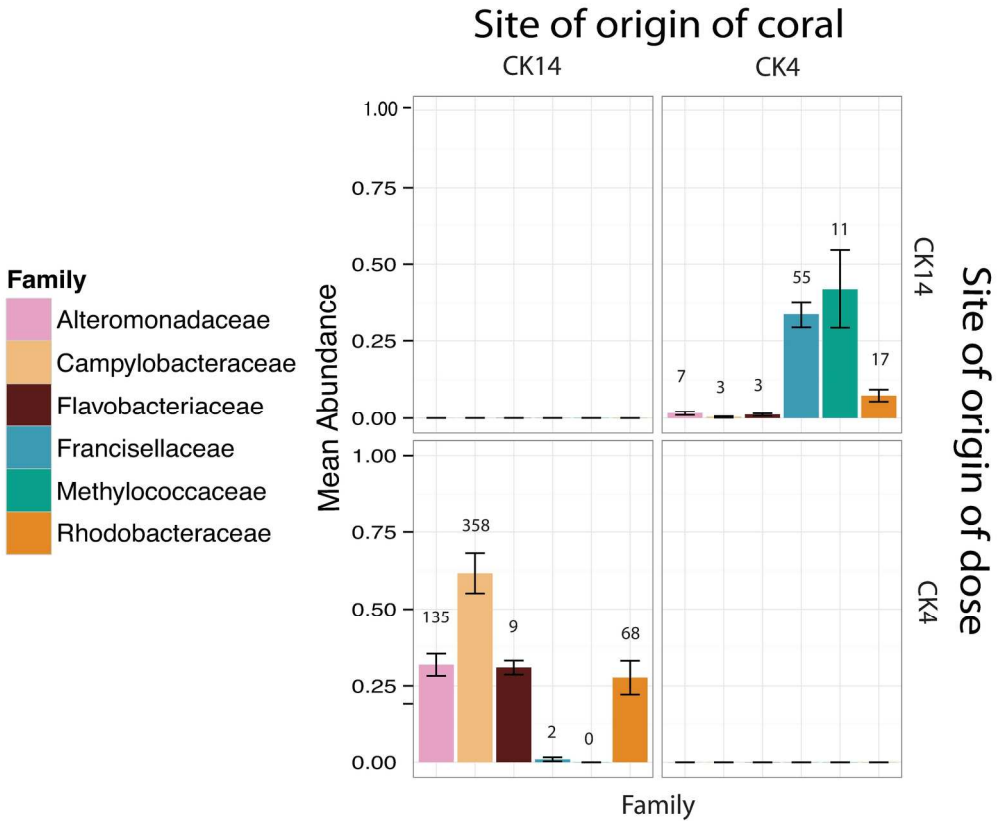

Fig. 3. Mean abundance of secondary OTUs belonging to selected families on dosed corals that became diseased at time three. Dosed corals are separated by the site of origin of the dose and the site of origin of the corals. OTUs are grouped by family, and the number of OTUs in each group is noted on the top of the mean abundance bar.

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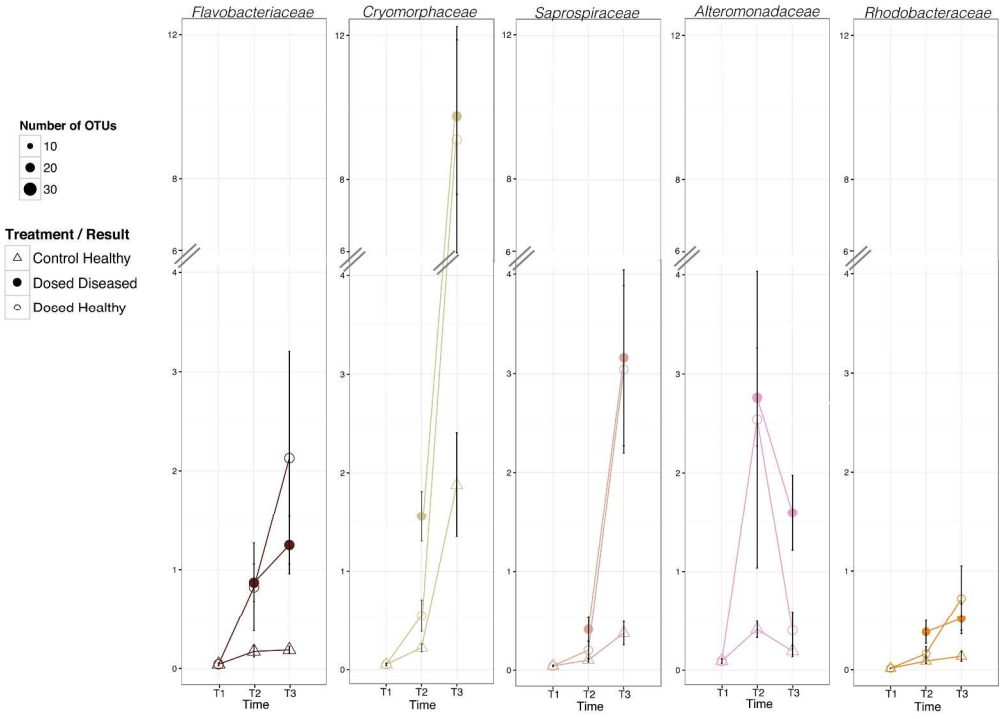


Fig. 4. Mean abundance of primary responders belonging to selected families across time. OTUs are grouped by family, and the size of the points denotes how many OTUs belonged to that family.

289x206mm (300 x 300 DPI)

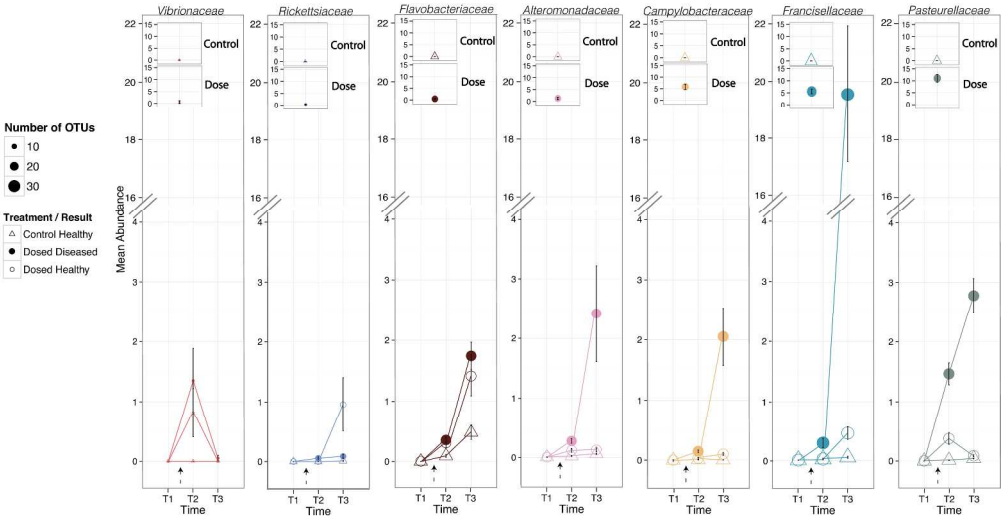
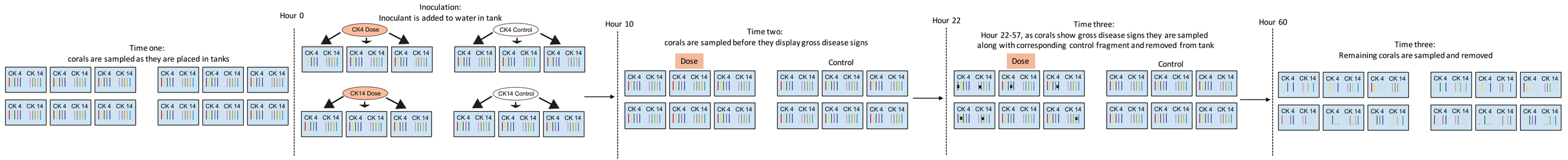


Fig. 5. Mean abundance of primary colonizers belonging to selected families across time. OTUs are grouped by family, and the size of the points denotes how many OTUs belonged to the specified family. Inset is the mean abundance for OTUs in that family in the inoculants (dose and control). Arrows signify time of inoculation. Error bars denote standard error.

309x160mm (300 x 300 DPI)

Table 1. PERMANOVA of Bray-Curtis dissimilarity between samples collected at times two and three.

Effect	df	Sums of Sqs	Mean Sqs	F Model	R^2	P
Final disease state	**1**	**3.49**	**3.49**	**9.43**	**0.030**	**0.001**
Inoculant	**1**	**1.06**	**1.06**	**2.86**	**0.0092**	**0.001**
Time	**1**	**7.77**	**2.59**	**7.00**	**0.067**	**0.001**
Inoculant site	**1**	**0.89**	**0.89**	**2.40**	**0.0077**	**0.001**
Inoculant x time	**1**	**1.67**	**0.83**	**2.25**	**0.014**	**0.001**
Inoculant x inoculant site	**1**	**1.20**	**1.20**	**3.23**	**0.010**	**0.001**
Time x inoculant site	**1**	**2.41**	**0.80**	**2.17**	**0.020**	**0.001**
Inoculant x time x inoculant site	**1**	**1.60**	**0.80**	**2.15**	**0.014**	**0.001**
Residuals	162	95.46	0.37		0.83	
Total	178	115.53			1.00	


Time one:
corals are sampled as they are placed in tanks
Hour 0
Inoculation:
Inoculant is added to water in tank
CK4 Dose
CK4 Control
CK14 Dose
CK14 Control
Hour 10
Time two:
corals are sampled before they display gross disease signs
Dose
Control
Hour 22
Time three:
Hour 22-57, as corals show gross disease signs they are sampled
along with corresponding control fragment and removed from tank
Dose
Control
Hour 60
Time three:
Remaining corals are sampled and removed
CK 4 CK 14

Tank	Colony	SiteInoculant	Site	Inoculant	FinalDiseaseState
CK14D1	B_CK14	CK14	CK14	Dose	Diseased
CK14D2	B_CK14	CK14	CK14	Dose	Diseased
CK14D3	B_CK14	CK14	CK14	Dose	Diseased
CK14H1	B_CK14	CK14	CK14	Control	Healthy
CK14H2	B_CK14	CK14	CK14	Control	Diseased
CK14H3	B_CK14	CK14	CK14	Control	Healthy
CK4D1	B_CK14	CK4	CK14	Dose	Diseased
CK4D2	B_CK14	CK4	CK14	Dose	Diseased
CK4D3	B_CK14	CK4	CK14	Dose	Diseased
CK4H1	B_CK14	CK4	CK14	Control	Healthy
CK4H2	B_CK14	CK4	CK14	Control	Healthy
CK4H3	B_CK14	CK4	CK14	Control	Healthy
CK14D1	B_CK4	CK14	CK4	Dose	Healthy
CK14D2	B_CK4	CK14	CK4	Dose	Diseased
CK14D3	B_CK4	CK14	CK4	Dose	Healthy
CK14H1	B_CK4	CK14	CK4	Control	Healthy
CK14H2	B_CK4	CK14	CK4	Control	Healthy
CK14H3	B_CK4	CK14	CK4	Control	Healthy
CK4D1	B_CK4	CK4	CK4	Dose	Diseased
CK4D2	B_CK4	CK4	CK4	Dose	Healthy
CK4D3	B_CK4	CK4	CK4	Dose	Diseased
CK4H1	B_CK4	CK4	CK4	Control	Healthy
CK4H2	B_CK4	CK4	CK4	Control	Healthy
CK4H3	B_CK4	CK4	CK4	Control	Healthy
CK14D1	G_CK14	CK14	CK14	Dose	Diseased
CK14D2	G_CK14	CK14	CK14	Dose	Diseased
CK14D3	G_CK14	CK14	CK14	Dose	Healthy
CK14H1	G_CK14	CK14	CK14	Control	Healthy
CK14H2	G_CK14	CK14	CK14	Control	Healthy
CK14H3	G_CK14	CK14	CK14	Control	Healthy
CK4D1	G_CK14	CK4	CK14	Dose	Healthy
CK4D2	G_CK14	CK4	CK14	Dose	Diseased
CK4D3	G_CK14	CK4	CK14	Dose	Diseased
CK4H1	G_CK14	CK4	CK14	Control	Healthy
CK4H2	G_CK14	CK4	CK14	Control	Healthy
CK4H3	G_CK14	CK4	CK14	Control	Healthy
CK14D1	G_CK4	CK14	CK4	Dose	Diseased
CK14D2	G_CK4	CK14	CK4	Dose	Diseased
CK14D3	G_CK4	CK14	CK4	Dose	Healthy
CK14H1	G_CK4	CK14	CK4	Control	Healthy

CK14H2	G_CK4	CK14	CK4	Control	Healthy
CK14H3	G_CK4	CK14	CK4	Control	Healthy
CK4D1	G_CK4	CK4	CK4	Dose	Diseased
CK4D2	G_CK4	CK4	CK4	Dose	Diseased
CK4D3	G_CK4	CK4	CK4	Dose	Diseased
CK4H1	G_CK4	CK4	CK4	Control	Healthy
CK4H2	G_CK4	CK4	CK4	Control	Healthy
CK4H3	G_CK4	CK4	CK4	Control	Healthy
CK14D1	O_CK14	CK14	CK14	Dose	Healthy
CK14D2	O_CK14	CK14	CK14	Dose	Diseased
CK14D3	O_CK14	CK14	CK14	Dose	Healthy
CK14H1	O_CK14	CK14	CK14	Control	Healthy
CK14H2	O_CK14	CK14	CK14	Control	Healthy
CK14H3	O_CK14	CK14	CK14	Control	Healthy
CK4D1	O_CK14	CK4	CK14	Dose	Diseased
CK4D2	O_CK14	CK4	CK14	Dose	Diseased
CK4D3	O_CK14	CK4	CK14	Dose	Diseased
CK4H1	O_CK14	CK4	CK14	Control	Diseased
CK4H2	O_CK14	CK4	CK14	Control	Healthy
CK4H3	O_CK14	CK4	CK14	Control	Healthy
CK14D1	O_CK4	CK14	CK4	Dose	Healthy
CK14D2	O_CK4	CK14	CK4	Dose	Diseased
CK14D3	O_CK4	CK14	CK4	Dose	Healthy
CK14H1	O_CK4	CK14	CK4	Control	Healthy
CK14H2	O_CK4	CK14	CK4	Control	Healthy
CK14H3	O_CK4	CK14	CK4	Control	Healthy
CK4D1	O_CK4	CK4	CK4	Dose	Diseased
CK4D2	O_CK4	CK4	CK4	Dose	Diseased
CK4D3	O_CK4	CK4	CK4	Dose	Diseased
CK4H1	O_CK4	CK4	CK4	Control	Healthy
CK4H2	O_CK4	CK4	CK4	Control	Healthy
CK4H3	O_CK4	CK4	CK4	Control	Healthy
CK14D1	P_CK14	CK14	CK14	Dose	Healthy
CK14D2	P_CK14	CK14	CK14	Dose	Healthy
CK14D3	P_CK14	CK14	CK14	Dose	Healthy
CK14H1	P_CK14	CK14	CK14	Control	Healthy
CK14H2	P_CK14	CK14	CK14	Control	Healthy
CK14H3	P_CK14	CK14	CK14	Control	Healthy
CK4D1	P_CK14	CK4	CK14	Dose	Diseased
CK4D2	P_CK14	CK4	CK14	Dose	Diseased
CK4D3	P_CK14	CK4	CK14	Dose	Diseased

CK4H1	P_CK14	CK4	CK14	Control	Healthy
CK4H2	P_CK14	CK4	CK14	Control	Healthy
CK4H3	P_CK14	CK4	CK14	Control	Healthy
CK14D1	P_CK4	CK14	CK4	Dose	Diseased
CK14D2	P_CK4	CK14	CK4	Dose	Diseased
CK14D3	P_CK4	CK14	CK4	Dose	Healthy
CK14H1	P_CK4	CK14	CK4	Control	Healthy
CK14H2	P_CK4	CK14	CK4	Control	Healthy
CK14H3	P_CK4	CK14	CK4	Control	Healthy
CK4D1	P_CK4	CK4	CK4	Dose	Diseased
CK4D2	P_CK4	CK4	CK4	Dose	Diseased
CK4D3	P_CK4	CK4	CK4	Dose	Diseased
CK4H1	P_CK4	CK4	CK4	Control	Healthy
CK4H2	P_CK4	CK4	CK4	Control	Healthy
CK4H3	P_CK4	CK4	CK4	Control	Healthy
CK14D1	W_CK14	CK14	CK14	Dose	Diseased
CK14D2	W_CK14	CK14	CK14	Dose	Healthy
CK14D3	W_CK14	CK14	CK14	Dose	Healthy
CK14H1	W_CK14	CK14	CK14	Control	Healthy
CK14H2	W_CK14	CK14	CK14	Control	Healthy
CK14H3	W_CK14	CK14	CK14	Control	Healthy
CK4D1	W_CK14	CK4	CK14	Dose	Diseased
CK4D2	W_CK14	CK4	CK14	Dose	Diseased
CK4D3	W_CK14	CK4	CK14	Dose	Diseased
CK4H1	W_CK14	CK4	CK14	Control	Healthy
CK4H2	W_CK14	CK4	CK14	Control	Healthy
CK4H3	W_CK14	CK4	CK14	Control	Healthy
CK14D1	W_CK4	CK14	CK4	Dose	Diseased
CK14D2	W_CK4	CK14	CK4	Dose	Healthy
CK14D3	W_CK4	CK14	CK4	Dose	Healthy
CK14H1	W_CK4	CK14	CK4	Control	Healthy
CK14H2	W_CK4	CK14	CK4	Control	Healthy
CK14H3	W_CK4	CK14	CK4	Control	Healthy
CK4D1	W_CK4	CK4	CK4	Dose	Diseased
CK4D2	W_CK4	CK4	CK4	Dose	Diseased
CK4D3	W_CK4	CK4	CK4	Dose	Diseased
CK4H1	W_CK4	CK4	CK4	Control	Healthy
CK4H2	W_CK4	CK4	CK4	Control	Healthy
CK4H3	W_CK4	CK4	CK4	Control	Healthy

Time_diseased	Time_survived	
	37	37
	37	37
	37	37
N/A		60
	50	50
N/A		60
	50	50
	50	50
	22	22
N/A		60
N/A		60
N/A		60
N/A		60
	57	57
N/A		60
N/A		60
N/A		60
N/A		60
	50	50
N/A		60
	22	22
N/A		60
N/A		60
N/A		60
	57	57
	57	57
NA		60
N/A		60
N/A		60
N/A		60
N/A		60
	34	34
	34	34
N/A		60
N/A		60
N/A		60
	57	57
	57	57
N/A		60
N/A		60

N/A		60
N/A		60
	57	57
	50	50
	22	22
N/A		60
N/A		60
N/A		60
N/A		60
	57	57
N/A		60
N/A		60
N/A		60
N/A		60
	57	57
	34	34
	34	34
	50	50
N/A		60
N/A		60
N/A		60
	37	37
N/A		60
N/A		60
N/A		60
N/A		60
	37	37
	37	37
	22	22
N/A		60
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N/A		60
N/A		60
N/A		60
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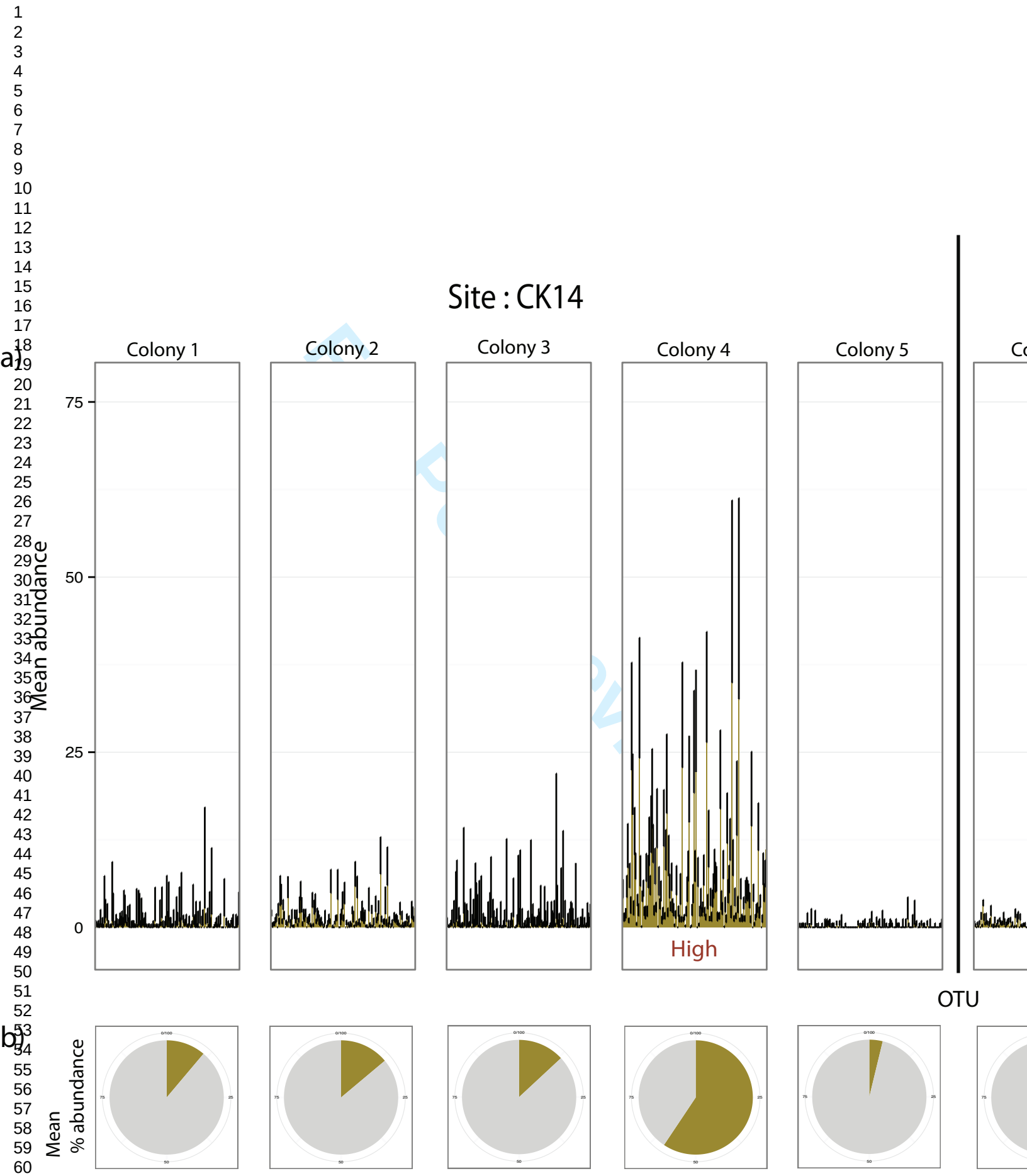
N/A		60
N/A		60
N/A		60
	50	50
	50	50
	34	34

N/A		60
N/A		60
N/A		60
	57	57
	57	57
N/A		60
N/A		60
N/A		60
N/A		60
	57	57
	34	34
	34	34
N/A		60
N/A		60
N/A		60
	37	37
N/A		60
N/A		60
N/A		60
N/A		60
N/A		60
	34	34
	50	50
	22	22
N/A		60
N/A		60
N/A		60
	57	57
N/A		60
N/A		60
N/A		60
N/A		60
N/A		60
	50	50
	34	34
	22	22
N/A		60
N/A		60
N/A		60

Supplementary Table 2. Mean diversity of bacterial communities associated with groups of coral.

Time, Inoculant, Final disease state	Shannon	SE	Richness	SE
Inoculant, Dose, Diseased	3.83	0.12	400.40	47.77
Three, Dose, Diseased	4.18	0.19	402.57	21.38
Two, Dose, Diseased	3.62	0.15	333.42	15.28
Three, Dose, Healthy	3.16	0.35	326.96	35.85
Two, Dose, Healthy	3.16	0.39	309.52	41.93
Inoculant, Control, Healthy	3.09	0.33	279.55	19.63
One, Control, Healthy	2.13	0.12	224.43	13.47
Three, Control, Healthy	3.26	0.19	345.90	20.47
Two, Control, Healthy	3.00	0.13	274.16	12.14


a)
Site : CK14
Colony 1
Colony 2
Colony 3
Colony 4
Colony 5
Mean abundance
75
50
25
0
High
OTU
b)
Mean % abundance