Final report. Submitted by Dr. Sean Callahan on 3/30/15

A. **Award Number**: NA12NOS4820070

B. **Project Title:** Step two in the rapid response investigation of an outbreak of acute *Montipora* white syndrome in Hawaii: outbreak impacts and disease etiology and pathogenesis.

C. **Total Amount of Award:** $124,000.00
Federal funds: $62,000.00 ($49,600.00 direct, $12,400.00 indirect); Matching funds: $62,000.00

D. **Federal Funds received to date:** $62,000.00

E. **Award Period:** 7/01/12-12/31/14 with one-year no-cost extension

F. **Period Covered by this Report:** 7/01/12-12/31/14 - Final report

G. **Summary**

In March, 2010 and subsequently in January, 2012 disease outbreaks on the reefs of Kaneohe Bay, Oahu of acute *Montipora* white syndrome (aMWS) caused tissue loss and the subsequent death of hundreds of coral colonies. Hawaii's rapid response team surveyed the damage and collected samples for follow-up research. This project represents the follow-up of the initial response to the disease outbreaks, which consisted of quantification of the effect of the disease outbreaks on the coral reefs and investigation of the pathogenesis and etiology of the disease. The project had six objectives that were completed essentially as described in the proposal. Each objective is listed below with a summary of results and conclusions.

1. Conduct quantitative surveys to determine the level (# affected colonies/m^2 host coral) and incidence (change in disease levels through time) of acute *Montipora* white syndrome on 12 semi-permanent sites surveyed during the original outbreak.

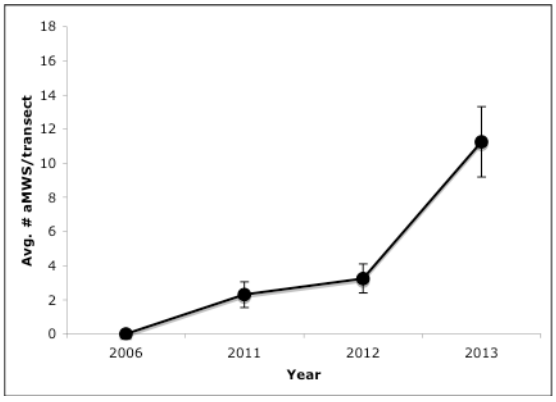

Figure 1. Changes in levels of aMWS at 12 semi-permanent sites around Coconut Island, Kaneohe Bay, Oahu.

In 2006, no cases of aMWS were found when the 12 semi-permanent sites around Coconut Island were initially established. All sites were resurveyed in March 2013 and 135 cases were documented. This is higher than prior surveys at these same sites (Fig. 1) and higher than levels found during the original outbreak in March 2010 (total # of aMWS=51). This suggests that aMWS is increasing in *M. capitata* populations on reefs surrounding Coconut Island.

During this same time period the amount of affected host coral (*M. capitata*) found at these sites had an average decline from 32.8% cover in 2006 to 26.5% cover in 2013 (Fig. 2). The change in coral cover varied among the sites surveyed with 10 sites showing a decline in *M. capitata* cover and 2 sites showing an increase in cover (Table 1).

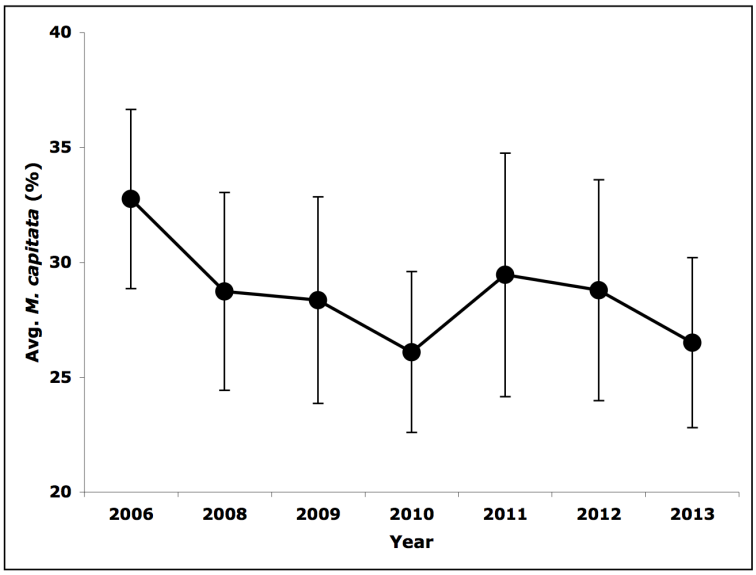

Figure 2. Changes in *M. capitata* cover through time at 12 semi-permanent sites around Coconut Island, Kaneohe Bay, Oahu.

transect	% *M. capitata* 2006	% *M. capitata* 2013	Change in % *M. capitata*
A	16	8.9	-7.1
B	29	19.8	-9.2
C	44	40.6	-3.4
D	59	49.5	-9.5
G	26	5.9	-20.1
H	12	18.8	6.8
I	31	22.8	-8.2
J	21	25.7	4.7
E	38	31.7	-6.3
F	44	40.6	-3.4
K	30	25.7	-4.3
L	43	28	-15

Table 1. Change in *M. capitata* cover through time at 12 semi-permanent sites around Coconut Island, Kaneohe Bay, Oahu.

2. Conduct rapid visual surveys on the same fringing and patch reefs as the original disease response to determine current distribution of acute MWS within Kaneohe Bay.

Rapid visual surveys of Kaneohe Bay were conducted in March of 2013. Within Kaneohe Bay we found a total of 851 colonies of *M. capitata* affected by aMWS. This is almost 3x as many colonies as discovered during the original 2010 outbreak (total=334) but less than that found during the 2012 outbreak (Fig. 3). South Kaneohe Bay had higher levels of aMWS (851 colonies) as compared to Central Kaneohe Bay (87 colonies) and north Kaneohe Bay (41 colonies), which is consistent with surveys conducted during both past outbreak periods.

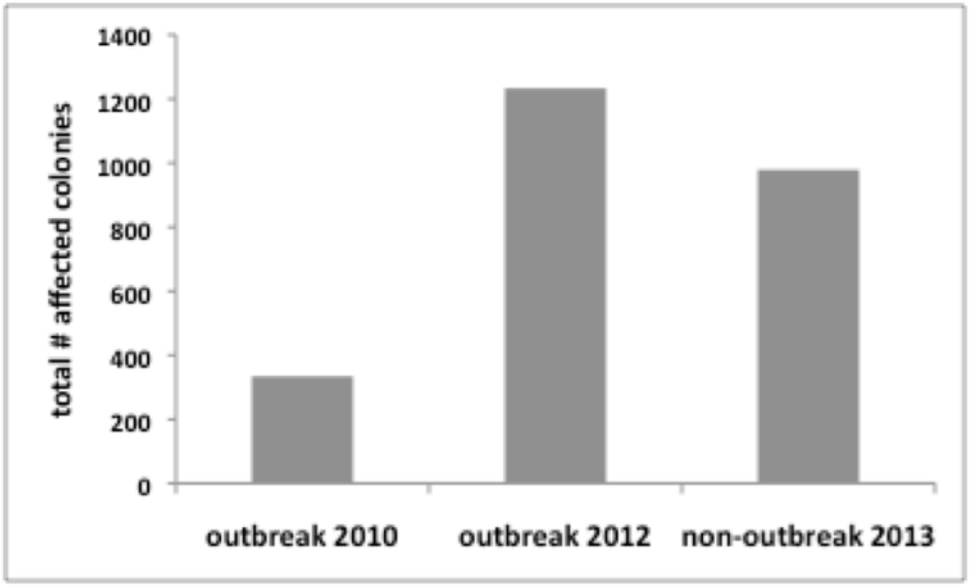

Figure 3. Total number of colonies of *M. capitata* affected by aMWS within Kaneohe Bay through time.

3. Compare the microbial communities of healthy coral (*M. capitata*) with coral exhibiting acute MWS.

Bacteria from seven samples each of healthy colonies, the healthy portion of diseased colonies (healthy-diseased) and lesions from *M. capitata* colonies exhibiting aMWS (diseased) coral collected during the 2012 outbreak were determined by sequencing of full 16S rRNA genes. The roughly 35,000 sequences of suitable quality were grouped into operational taxonomic units (OTUs) that were each comprised of sequences that shared 97% identity. The most striking finding was that bacteria of the family **Enterobacteraceae** dominated all samples (Fig. 4). Members of the Enterobacteraceae are typically found in the gastrointestinal tract of mammals and in soils. Their abundance in a seawater sample is often used as an indicator of sewage contamination and water quality in general. Remarkably, a single OTU represented 84% of all sequences and 86% of healthy, 85% of healthy-diseased and 82% of diseased coral (Table 2). This single OTU was most similar overall to the bacterium *Shigella sonnei*, which is an

inhabitant of the human gastrointestinal tract, is present in feces, and is the causative agent of Shigellosis, an infection of the intestinal mucosa. Several bacteria of the Enterobacteraceae found in large numbers in human feces were represented in this dominant OTU (Table 3).

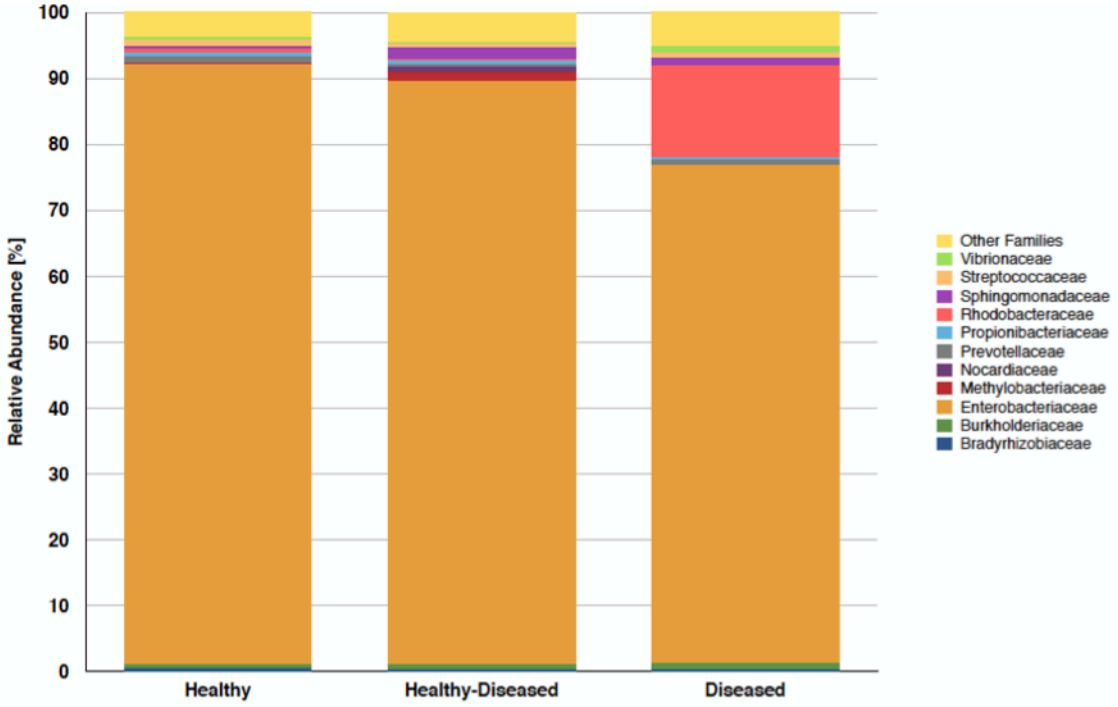

Figure 4. Comparison of types and abundance of bacteria in samples from healthy *M. capitata* (healthy), healthy portion of diseased colonies (healthy-diseased) and the lesions from colonies exhibing aMWS (diseased).

Cluster	Blast Classification	Identity [%]	Abundance [%]: Healthy	Abundance [%]: Healthy-Diseased	Abundance [%]: Diseased
OTU 001	*Shigella sonnei*	97	86.04	84.69	81.99
OTU 002	*Nautella italica*	97	1.19	3.50	6.50
OTU 003	*Sphingomonas pituitosa*	94	0.43	1.47	1.21
OTU 004	*Methylobacterium oryzae*	97	0.31	0.81	0.68
OTU 005	*Streptococcus sanguinis*	99	0.39	0.50	0.71
OTU 006	*Vibrio brasiliensis*	99	0.37	0.48	0.54
OTU 007	*Bacteroides acidifaciens*	88	0.34	0.44	0.47
OTU 008	*Propionibacterium acnes*	98	0.33	0.38	0.41
OTU 009	*Rhodococcus rhodnii*	94	0.31	0.37	0.39
OTU 010	*Ralstonia picketti*	99	0.30	0.32	0.33

Table 2. Identification and abundance (proportion of all gene sequences identified) of the common bacteria found in samples from healthy *M. capitata* (healthy), healthy portion of diseased colonies (healthy-diseased) and the lesions from colonies exhibiting aMWS (diseased).

Blast Result for OTU 001	Identity [%]
Shigella sonnei	97
Shigella flexneri	97
Escherichia fergusonii	97
Shigella boydii	97
Escherichia coli	97
Escherichia albertii	97
Shigella dysenteriae	97
Escherichia vulneris	97

Table 3. Identification of bacteria within the dominant OTU (Enterobacteraceae) found in samples of *M. capitata* collected during the 2010 aMWS outbreak within Kaneohe Bay.

We also found an increased abundance of bacteria of the family **Rhodobacteraceae** from the lesions of colonies exhibiting signs of aMWS as compared to,the healthy portion of aMWS colonies (healthy-disease) or from healthy coral (Fig. 4). Increases in Rhodobacteraceae have been observed in increased numbers specifically in diseased coral before (Mouchka et al., 2010).

At a lower taxonomic level, differences in the bacterial communities between healthy, healthy-diseased and diseased colonies were visualized using principle coordinate analysis. There was high overlap among sample types with one outlier (D2) evident in the diseased samples (Fig. 5). This indicates that communities from different sample types are not significantly different when only the presence or absence of different bacteria in each community is considered. This finding is also reflected in the parsimony and unweighted UniFrac analysis (Table 4). However, weighted UniFrac analysis, which takes into account the abundance as well as the presence of each type of bacterium, indicated that community structures were significantly different (Table 4).

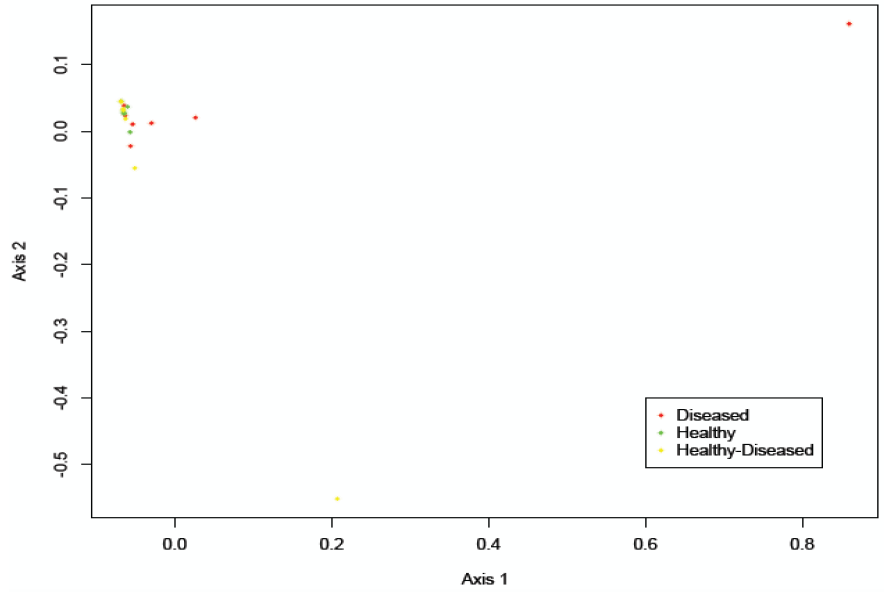

Figure 5. Principal coordinate analysis showing differences in bacterial communities within and between coral samples from healthy coral, healthy portion of diseased coral and colonies exhibiting aMWS (diseased).

Groups	Parsimony Score	P-value	UniFrac (weighted)	P-value	UniFrac (unweighted)	P-value
Healthy — Diseased	3	0.057	0.153524	<0.001*	0.513344	0.651
Healthy-Diseased — Diseased	5	0.705	0.153818	<0.001*	0.631867	0.7
Healthy — Healthy-Diseased	5	0.676	0.15095	<0.001*	0.510209	0.762

Table 4. Differences in bacterial communities between coral samples from healthy, healthy portion of diseased coral and colonies exhibiting aMWS (diseased). Weighted UniFrac analyses examine presence and abundance of types of bacteria in samples whereas unweighted UniFrac only represents differences in types of bacteria present in the different coral samples.

4. Conduct challenge experiments to determine if culturable bacteria cause acute MWS.

None of the 265 bacterial strains cultured from diseased coral collected during the 2010 outbreak consistently recreated disease in laboratory infection trials, suggesting that bacteria that caused the outbreak were not cultivable on the media used here. However, three strains were identified as *Pseudoalteromonas* by16S rRNA sequencing as well as multi-locus sequence analysis. These bacteria are very similar to another strain of *Pseudoalteromonas*, strain OCN003, which we had previously isolated from diseased *M. capitata* and found that it recreated signs of acute tissue loss (aMWS) in laboratory infection trials at a low rate; about 25% of fragments show signs of disease in infection trials. Because all three strains were genetically similar, one strain (OCN050) was chosen to represent all. OCN050 is not likely to be a primary pathogen because it has a low infection rate similar to that of OCN003. However, when strain OCN050 was used as a secondary pathogen on corals that were already compromised by chronic *Montipora* White syndrome (cMWS) infection rates were higher (Fig. 6 and 7). cMWS is a less virulent, much slower moving tissue loss disease that is prevalent in Kaneohe Bay (Aeby et al. 2010). In contrast, another bacterium known to cause aMWS on healthy coral, *Vibrio coralliilyticus* strain OCN008 (Ushijima et al., 2014), was unsuccessful in causing a similar shift from cMWS to aMWS.

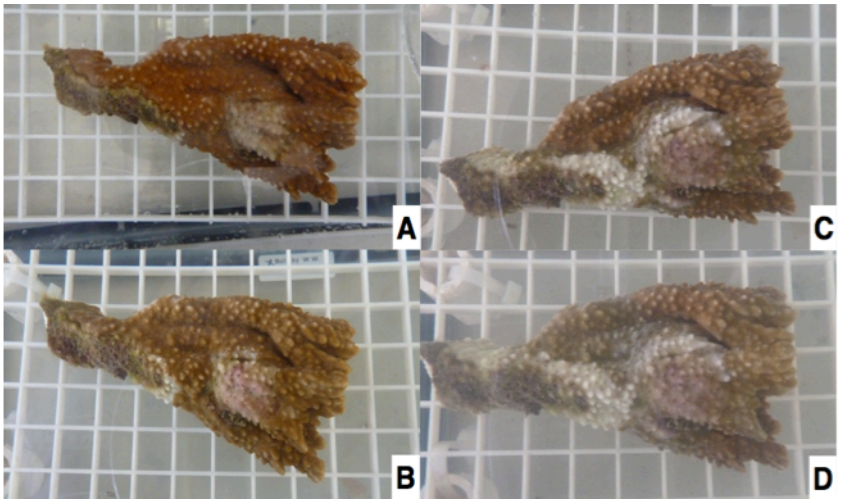

Figure 6. Chronic MWS fragment inoculated with OCN050. (A) *Montipora capitata* fragment exhibiting chronic MWS before inoculation with OCN050. (B) Fragment three days post-inoculation, lesion switched from chronic to acute. (C) Seven days after inoculation, approximately half of the fragment exhibited acute tissue loss. (D) After eight days, the fragment had little remaining healthy tissue.

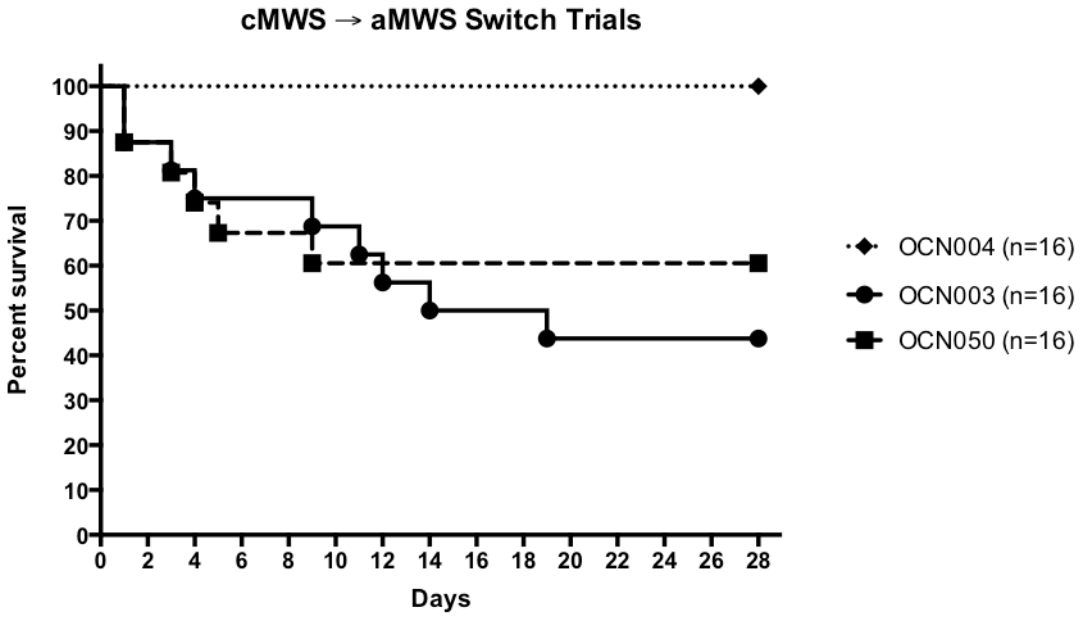

Figure 7. Survivorship plot of *M. capitata* fragments exhibiting signs of cMWS that switched to acute tissue loss (aMWS) when exposed to different bacteria. Strain OCN004 is a strain of *Alteromonas* that serves as a negative control. Strain OCN003 is a proposed secondary pathogen of *M. capitata* isolated from diseased *M. capitata* in a prior study. OCN050 is a potential secondary pathogen isolated from diseased *M. capitata* collected during the 2010 disease outbreak in Kaneohe Bay.

5. Conduct manipulative experiments to determine environmental co-factors involved in the development of acute MWS.

Controlled laboratory experiments were used to determine whether elevated temperature (28 ° C), nutrient stress (nitrate) or a combination of nitrate plus elevated temperature affected the progression of chronic MWS into the acute phase of disease (aMWS). There was no consistent effect of elevated temperature, nutrient stress or the combination of nitrate plus elevated temperature on the proportion of fragments that switched from chronic *Montipora* white syndrome (cMWS) to acute *Montipora* white syndrome (aMWS) (Fig. 8A, B, C). Both experimental and control fragments switched to aMWS during the experiments but there was a trend for more fragments exposed to nitrate or nitrate plus elevated temperature to switch. Now that these two environmental factors have been eliminated as major triggers for the disease outbreaks, other factors such as cold stress, salinity stress or sewage contamination should be examined.

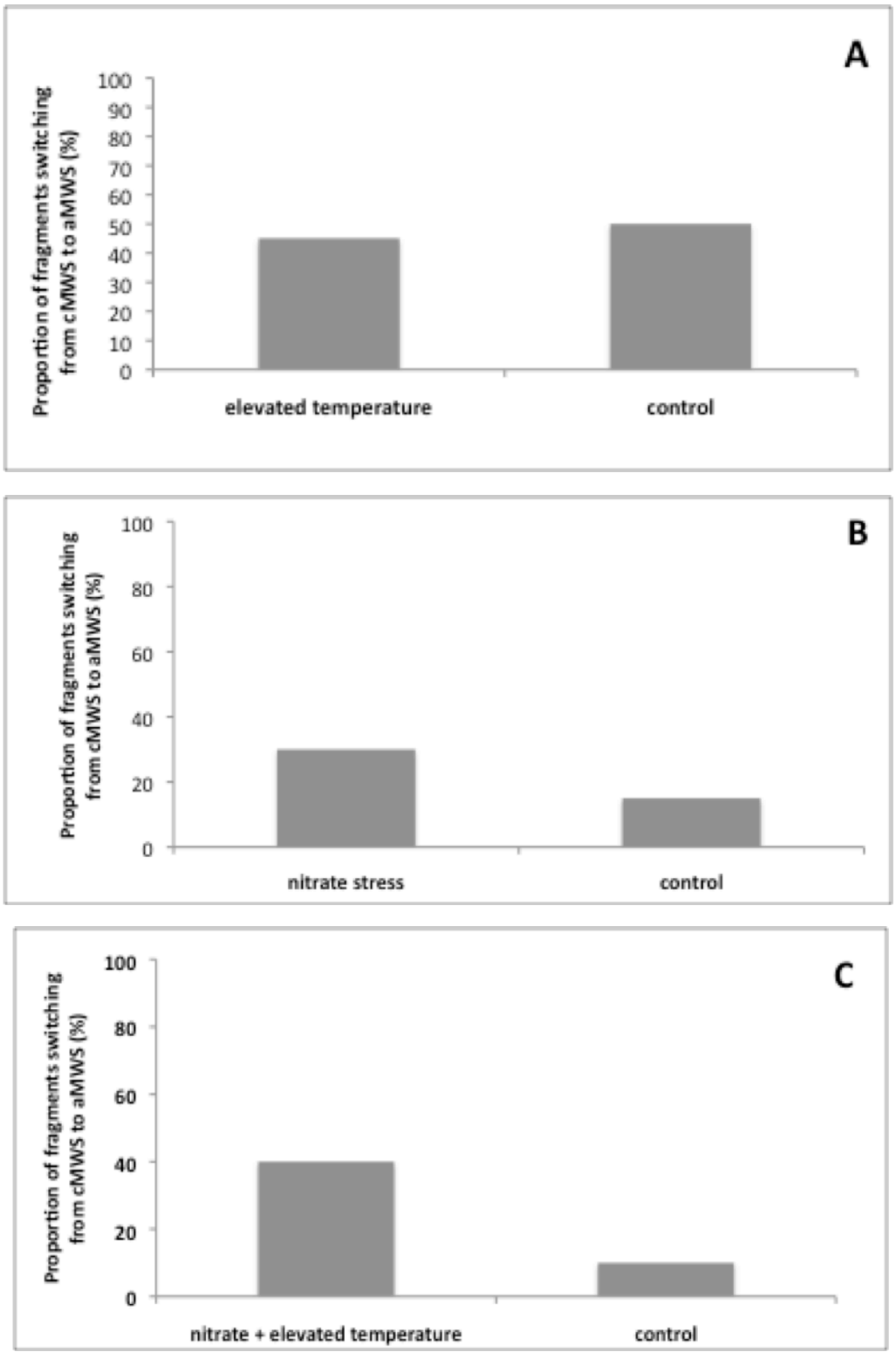

Figure 8. Proportion of *M. capitata* fragments that switched from cMWS to aMWS under experimental conditions (n=20 fragments/experiment). A) Experimental temperature ~28C and control temperature ~ 26C. B) nitrate stress ~ 10µm. C) nitrate stress~10µm plus experimental temperature ~28C.

6. Determine whether surgical removal is an effective treatment to control cMWS.

Experimental manipulations were conducted on the fringing reef of Moku o Loʻe in Kāneʻohe Bay. Twenty *Montipora capitata* colonies with chronic *Montipora* White Syndrome (cMWS) were tagged, and photographed. Ten colonies had the lesion removed (treatment) and ten colonies were left untreated as controls. All colonies were examined for progressive tissue loss or development of new lesions and photographed through time (Fig. 9). The complex structure of the coral colonies prevented use of digital measurement for rate of tissue loss. Hence, *in-situ* observations on the proportion of the colony that was healthy, diseased or dead was recorded at each survey period. Colonies were followed for 26 weeks post-lesion removal.

Removal of the active cMWS lesion resulted in a reduction in the average loss of healthy tissue from colonies (Mann-Whitney U, n=16, p=0.058). After 26 weeks, untreated colonies lost an average of 49% more tissue compared to treated colonies (Fig. 10). However, treatment did not prevent re-infection with disease re-occurring in colonies by week two post-treatment and continuing through time (Fig. 11). Active lesions appeared and disappeared through time on colonies in both groups of colonies (treated and control), which is consistent with other studies of cMWS (Aeby et al. 2010, Work et al. 2012). Re-infections did not occur around the treatment margins but on other areas of the colony. Treatment margins appeared to heal and were covered with tissue by week 5.

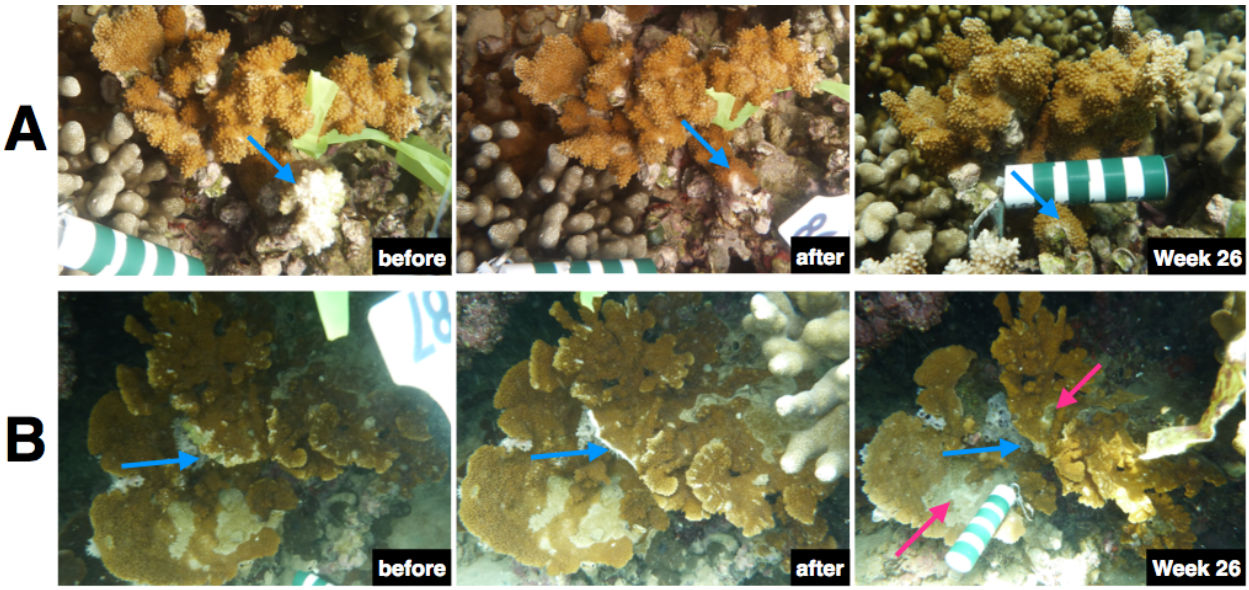

Fig. 9. (A) Example of a colony showing successful disease treatment. Blue arrows indicate lesions from disease or surgical removal. Panel on left shows diseased branch, middle panel shows the area after removal and panel on the right shows healed lesion from removal with no further evidence of disease 26 weeks post-treatment.
(B) Example of a treated colony that developed a disease lesion in a new spot. Left panel shows the colony before treatment, middle panel shows the area after treatment and right panel shows healing of original removal lesion (blue arrow) and development of a new disease lesion elsewhere on the colony (pink arrow).

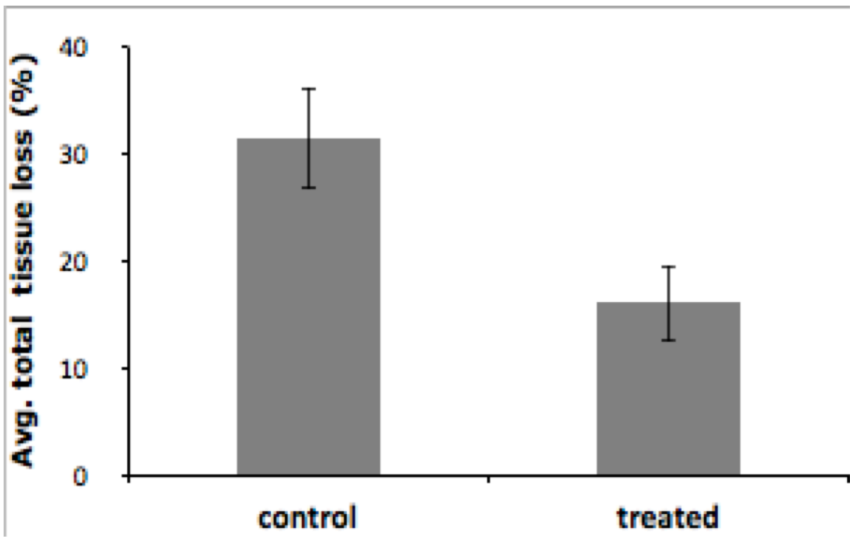

Figure 10. The effectiveness of lesion removal as a method of disease treatment for *M. capitata* colonies affected by cMWS. Data reflect mean and standard error of tissue loss after 26 weeks post-treatment (n=8/group).

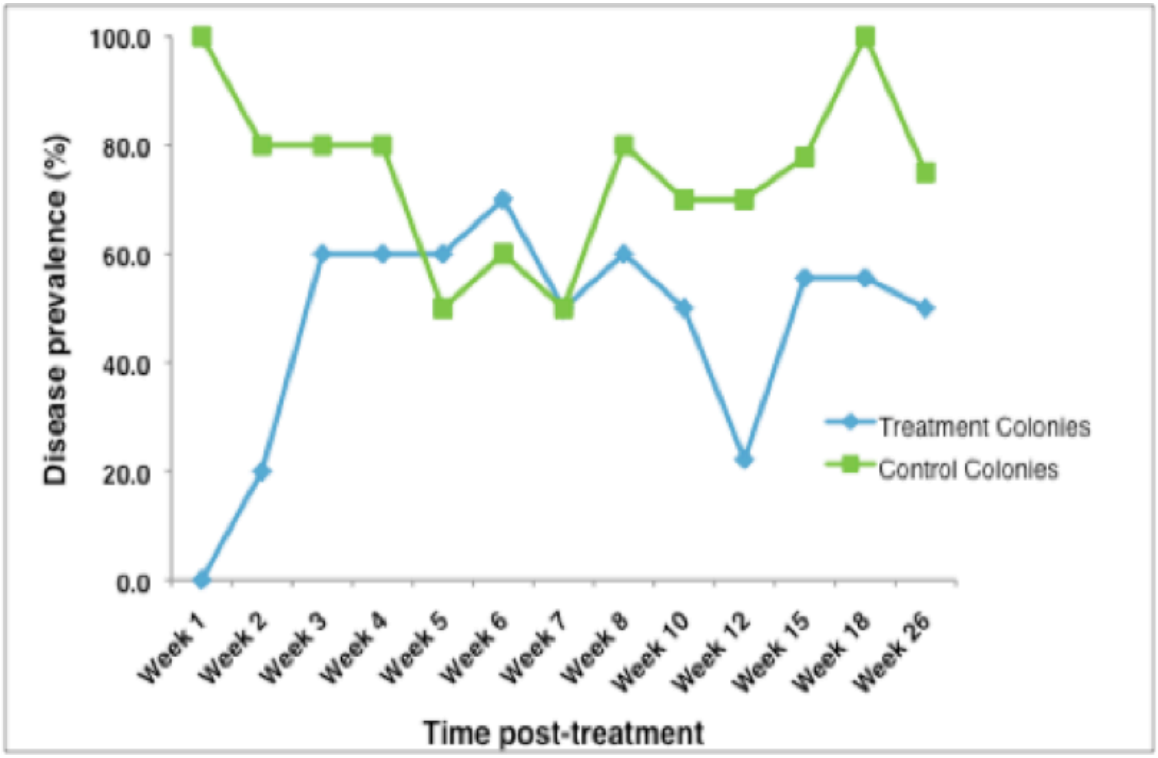

Figure 11. Prevalence of cMWS through time in *M. capitata* colonies that had the disease margin removed *in situ* (treatment colonies) or left in place (control colonies) (n=10* colonies/group).

*Treatment: n=10 until week 10, n=9 until week 18, n=8 until week 26, Control: n=10 until week 12, n=9 until week 15, n=8 until week 26.

Summary

- Levels of aMWS are increasing in Kaneohe Bay, and *M. capitata* cover is declining.
- Both the 2010 and 2012 outbreaks were preceded by large rain events. The predominance of Enterobacteracea in bacterial communities associated with healthy, healthy-diseased, and diseased *M. capitata* samples is consistent with colonization of coral colonies with bacteria derived from sewage and/or freshwater runoff.
- Strains of *Pseuodoalteromonas* were identified in diseased samples from the 2010 outbreak and were capable of causing a switch from cMWS to aMWS in the laboratory. However, these bacteria were unlikely to be the dominant cause of the outbreaks because they were not found in large numbers in the communities associated with diseased coral. Instead, bacteria from the family Rhodobacteracea were enhanced in the diseased samples. These bacteria require very specific culture techniques and would not be expected to be isolated using the general methods employed here, explaining why they were not represented in the infection trials. Follow-up studies on Rhodobacteracea as a potential etiological agent of acute tissue loss in *M. capitata* (aMWS) are recommended.
- Elevated seawater temperature, nutrient stress or the combination of nitrate plus elevated temperature, were eliminated as major triggers for the 2010 disease outbreak. There was no consistent effect of elevated temperature, nutrient stress or the combination of nitrate plus elevated temperature on the proportion of fragments that switched from cMWS to aMWS.
- Future studies should examine other environmental factors associated with heavy rain events such as cold stress, freshwater stress or sewage contamination.
- Surgical removal is an effective treatment to control cMWS but does not affect rates of re-infection on other parts of colonies.
- cMWS and aMWS are now common disease problems for *M. capitata* colonies in Kaneohe Bay. Treatment, such as surgical removal of disease lesions, would be more effective if used early to treat disease outbreaks before diseased colonies become prevalent.

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