2005 PROJECT SUMMARY

Project No:	**FGBNMS-WD05-May-RBJ**
Project Title:	*Comparison of bacterial communities among corals exhibiting white-syndrome (disease) signs*
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Grant Period:	NA
Project Start Date:	March 01, 2005
Project End Date:	ongoing
Location:	Flower Garden Banks National Marine Sanctuary

OBJECTIVES:

The objectives of this project focus on fundamental questions about a possible white-disease syndrome affecting hard corals on the reefs of the Flower Garden Banks National Marine Sanctuary (FGBNMS). While there has been a very low prevalence of white-disease syndrome on these reefs has been reported (and observed by RBJ of this project), a significant increase in prevalence was reported in February 2005. The exact nature of this white-disease syndrome outbreak was unknown. Because our research group was investigating white plague on reefs of St Croix (STX), US Virgin Islands and Lee Stocking Island (LSI), The Bahamas we were able to assemble a field sampling team along with necessary equipment to efficiently and effectively collect coral tissue samples from apparently diseased and apparently healthy coral colonies. Ultimately the objective of this work was directed toward establishing the cause of this white syndrome on corals of the FGBNMS. Functionally, by comparing bacterial communities on healthy and white-syndrome affected corals we hope to determine if there is a specific bacterium or consortium of bacteria that characterize the area of tissue loss. If unique and abundant bacteria are found along the margin of tissue loss, they could then be used to challenge healthy corals to assess their impact. At least superficially photographic evidence suggested the possibility that this white–syndrome might be white plague type II (WPII).

Our related white plague research is based on whether the causative agent(s) of WPII is an opportunistic pathogen(s) or an emerging, obligate pathogen or pathogens. For the purpose of that investigation we hypothesized that the causative agent, reported to be *Aurantimonas coralicida*, is an opportunistic pathogen normally present within the host coral or in the surrounding environment rather than a novel obligate pathogen. A major experimental objective was to compare corals exhibiting disease signs consistent with WPII from different geographical regions. Do they harbor the same community of microorganisms within their diseased tissues and, by extension, have the same etiology? We hypothesized that corals from two geographically distinct coral reefs exhibiting the same disease signs contain differing microbial communities and we are now adding the FGBNMS to that comparison. One specific objective of the FGBNMS study is to resolve the question of the nature of the white syndrome affecting the corals there. Does it fit the description of WPII? What ever the answer to this question, we will compare bacterial communities among these sites. This information will provide unique insight into the microflora of healthy corals, as well as apparently diseased corals, on a large regional scale.

Our previously stated objectives continue to lead to two specific experimental hypotheses:

H_{O1}: The causative agent of this white-disease syndrome, possibly WPII, is an opportunistic endemic organism that has become pathogenic

H_{A1}: The causative agent is a novel pathogen

H_{O2}: Corals affected by white-disease syndrome, possibly WPII from, different geographical regions contain the same bacterial communities

H_{A2}: Corals so affected from different geographical regions contain different bacterial communities

Our work focuses on **comparing the microbial community composition in healthy and diseased corals** using a combination of **molecular fingerprinting, microbial culture, and gene sequencing** to establish microbial community biodiversity and identifications in these corals, and by **comparative histological investigation** of the coral tissues to assess the underlying pathological changes associated with the gross disease signs. The report period described here (May 23-26, 2005) emphasized field observations and capturing images of white-disease syndrome affected corals and collection of high quality samples from both the East and West Banks of the FGBNMS. Samples were immediately plated on nutrient media to assess the culturable microbial community and, separately. preserved for molecular and histological analyses.

METHODS:

Sample Collection and Research Area Description

Samples were collected from both the East and West Banks of the FGBNMS during a research cruise from May 22 – 26, 2005 sponsored by the FGBNMS on board the M/V Fling (Figure 1). Dr. Jonas and Geoffrey M. Cook collected all samples. Essentially the same methods used in our previous coral disease work were employed. Samples of coral tissue and skeleton were collected in the same manner at all four sites. Colonies of *Montastraea faveolata*, *M. franksii*, *Colpophyllia natans*, and *Diploria strigosa* were sampled.

Figure 1. Map of FGBNMS

The sampling goal was to identify colonies exhibiting the most obvious signs of white-disease syndrome and to compare those signs with descriptions of WPII. A separate focus of the research cruise was to collect relatively large cores of coral. This effort dictated the actual mooring sites chosen during the cruise period. Corals with obvious active white-disease showing signs of WPII were located and sampled (Figure 2). At each collection station a visually disease-free colony of the same species was located near a white-disease affected colony. The "diseased" and "healthy" pair of colonies was then sampled. Coral tissue-plus-skeleton samples were collected using sterilized, stainless steel (ss) corers transported inside individual sterilized, screw-capped, conical-bottom polypropylene (pp) tubes filled with sterile seawater. The corers and tubes were handled with latex-gloved hands only.

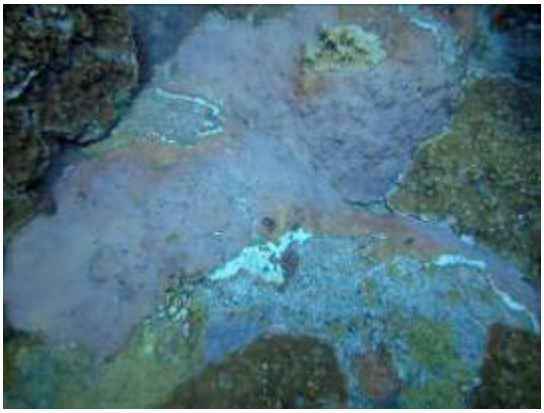


Figure 2. White-disease on *M. franksii* at Flower Garden Banks National Marine Sanctuary, May 2005

In order, five samples were collected from the apparently healthy colony, five from the apparently healthy portion of the diseased colony, and then five to eight from the disease interface on the diseased colony. This order was maintained to minimize potential contamination of healthy samples with material from diseased corals. In each case the five samples consisted of three (or six along the disease margin) 1.6-cm diameter core samples for molecular/microbiological analysis and two 2.4-cm diameter samples for histological analysis (Figure 3). The core samples were held inside the corers and returned to their respective pp tubes and capped for transport to the boat. Samples were either preserved for histological examination or held at ambient seawater temperature, in subdued light, until they were processed within a few hours of collection. Colonies were photographed for unique identification (Figure 4).

Figure 3. Set of samples for histology: from apparently healthy nearby colony (labeled with white tape), from healthy portion of diseased colony (yellow label), and from margin of active tissue loss (orange label) for STX.

Figure 4. *Montastraea faviolata* colony at East Bank of FGBNMS affected by white-disease.

Collection Schedule

The start date for this work was March 2005 when a rapid response to the apparent outbreak of white-disease was detected. Heavy winds and waves at FGBNMS delayed the cruise until late May 2005. We were able to locate coral colonies at both East and West Banks affected by the white-disease syndrome that had been previously described. Each sampled colony exhibited signs of active white-disease, which could potentially be WPII. We noted however that algal turf was often in close proximity to the disease-healthy tissue interface suggesting that the disease progression was relatively slow. We collected samples from pair of corals (healthy and diseased) during three days of the cruise.

May 23, 2005

- East Bank, **Buoy #3** = Dive #1 (search for active WPII-like infected colonies).
- East Bank, **Buoy #3** = Dive #2 (sampled *M. faviolata* colony infected with WPII-like disease). Samples EC301 diseased coral, EC306 apparently healthy coral. Tagged #19. Images archived.

May 24, 2005

- East Bank, **Buoy #3** = Dive #3 (returned to *M. faviolata* sampled during dive #2. Collected 2 additional samples [D6 and D7]. The colony is situated 120° off the U bolt of Buoy #3). Images archived.
- East Bank, **Buoy #4** = Dive #4 (sampled *M. faviolata* and tagged colony as #6). Samples EC401 diseased coral, EC406 apparently healthy coral. Images archived.
- East Bank, **Buoy #3** = Dive #5 (a colony of *C. natans* with WPII was located). No sampling occurred. Images archived.

May 25, 2005

- West Bank, **Buoy #1** = Dive #6 (sampled colony of *C. natans* with WPII-like disease). Tagged colony as #18. The colony lays 210° off the U bolt). Samples WC101 diseased coral, WC106 apparently healthy coral. No images collected.
- West Bank, **Buoy #2** = Dive #7 (sampled colony of *M. franksii* with WPII-like disease). Tagged colony as #11. The colony lays 150° off the U bolt. Samples WC201 diseased coral, WC206 apparently healthy coral. Images archived.

May 26, 2005

- West Bank, **Buoy #2** = Dive #8 (search for more diseased corals). Transported drilling equipment for other dive team.

- West bank, **Buoy #2** = Dive #9 (Applied experimental treatment to *M. franksii* colony sampled on dive #7 (Tag #11) (Palygorscite sepialite clay) A colony of *D. strigosa* was also sampled during this dive. WP was radiating from the middle of the colony outwards. There were obvious fish bites at the center of the affected area. There was no obvious algal turf on the denuded portion of the colony. Images archived.

Sample Processing

The 2.4-cm core samples for histological analyses were preserved immediately after collection. Seawater was gently decanted and the tube was filled with a solution of Z-Fix (1 part Z-Fix concentrate diluted with 4 parts of 36 ppt seawater prepared from 0.2 um porosity filtered sea water). The core tube remained in place to provide support for the coral disk during the return to the laboratory (Figure 5). For shipping back to GMU, strips of paper towels were put into the pp tubes to prevent movement during transport. The tubes were placed in sealable, plastic bags and carefully packed to prevent damage to the samples. On arrival at GMU, the ss core tubes and paper towel strips were removed (Figure 6).

Figure 5. Typical field preservation of histological samples. The coral sample is still within the corer that is in the tube.

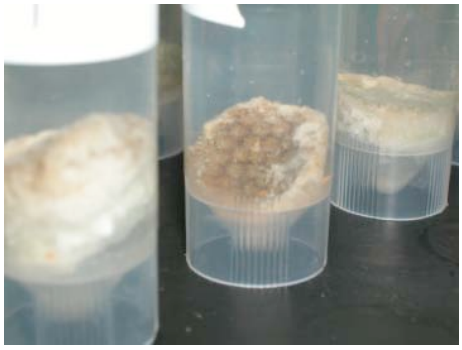


Figure 6. Examples of fixed coral cores after removal from the stainless steel corer.

Coral samples along with their associated skeletal material to be used for molecular/ microbial analysis were removed from the core tubes with a 10% bleach-rinsed (sterilized, depurinated) and sample water-rinsed steel rod (Figure 7). They were then immediately sectioned using a Dremel tool equipped with a diamond surfaced cutoff wheel (Figure 8). Healthy tissue samples were cut into two equal halves, one of which was placed in a cryotube for preservation by freezing. The edges of the "disease interface" samples were removed parallel to the interface to focus the analysis on the area of active tissue loss. This interface section was then frozen in a cryotube as well. This approach required that separate samples be collected for microbiological analysis. Molecular samples were frozen at -20 oC and then transferred to a dryshipper cooled with liquid nitrogen for shipping to GMU.

Figure 7 (left). Core of WP-like affected *M. faviolata* (EC301) coral tissue used for microbiological and molecular analyses.

Figure 8 (right). Using Dremel tool to trim and section coral core.

For microbiological analyses the "half-core" sections were crushed using bleach-rinsed, sample water-rinsed, ss, long-nosed pliers and diluted with 9.0 ml of sterile, 0.2 um porosity filtered sea water (Figure 9). The coral material was further homogenized with the bleach-rinsed steel rod and then vortexed at high speed for 2 minutes. Samples were then serially diluted in sterile seawater for microbiological analysis.

Figure 9 (left). Crushing the core with long-nosed pliers within the sample tube.

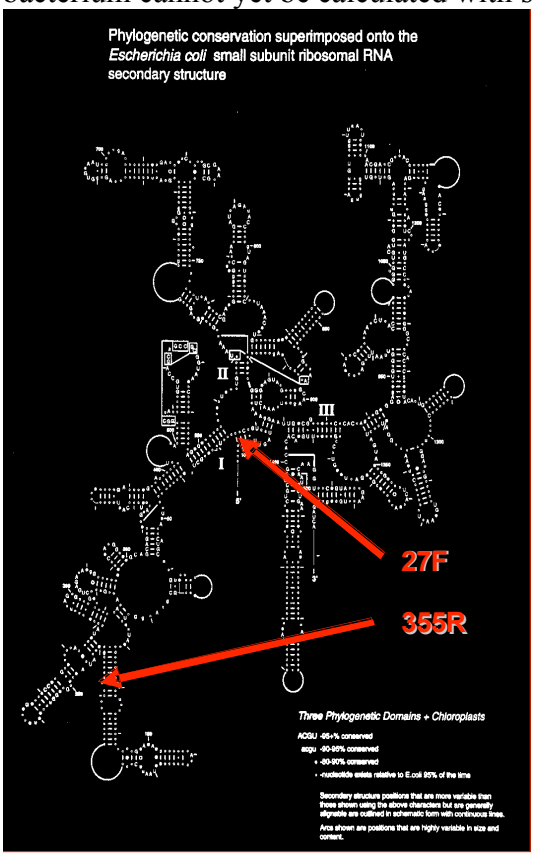
Aliquots of the diluted coral samples were plated on triplicate Petri dishes containing one-half strength Marine Agar 2216 (Zobell) made up at seawater salinity and 2% agar. This is a general medium for culturing marine bacteria. Additional agar is necessary to avoid spreading of highly motile marine bacteria. The medium was prepared in advance at GMU and allowed to dry in a laminar flow hood for three days prior to shipping to the collection sites, to reduce moisture on the surface of the plates. All plates were incubated at approximately 26°C for 5 – 7 days and then refrigerated at GMU to avoid overgrowth of bacterial colonies.

Sample Analysis

A combined protocol using both the BIO 101 FastDNA®Spin kit for Tissue and the BIO 101 FastDNA® Spin kit for Soil will be used to extract genomic DNA from the coral tissue samples. The Soil kit has not proven to be very efficient when used alone. However, combining the Tissue kit with the protein-binding matrix used in the Soil kit has been quite successful. It appears that the high quality preservation technique used here (rapid preservation at low temperature) resulted in inhibition of the PCR reaction (probably from mucopolysaccharides). The above combined technique was used to overcome this limitation. Good PCR products have been achieved very predictably for all of the samples collected from STX and LSI coral tissue

samples. The details of this procedure will be published with the results of this research and should be very valuable to other coral researchers.

As an example of our experimental approach molecular fingerprints of the bacterial community from pairs of corals from STX and LSI were obtained using Length Heterogeneity (LH) as described in NURP funded research proposal. Genomic DNA, extracted using the modified Bio101 kit was amplified using universal bacterial primers 27F (fluorescently labeled) and 355R (Figure 10). The small subunit ribosomal DNA (SSU rDNA) was separated with denaturing polyacrylamide gels using the SCE9610 fluorescent sequencer. These fingerprints, based on varying base-pair lengths of natural SSU rDNA fragments, provide identifying information, operational taxonomic units (OTU) and relative abundance estimates for members of the community. Fingerprints were compared among healthy colonies, and healthy areas and diseased areas on diseased colonies from STX and LSI. LH fingerprints for any individual bacterium cannot yet be calculated with sufficient precision to determine if a particular known



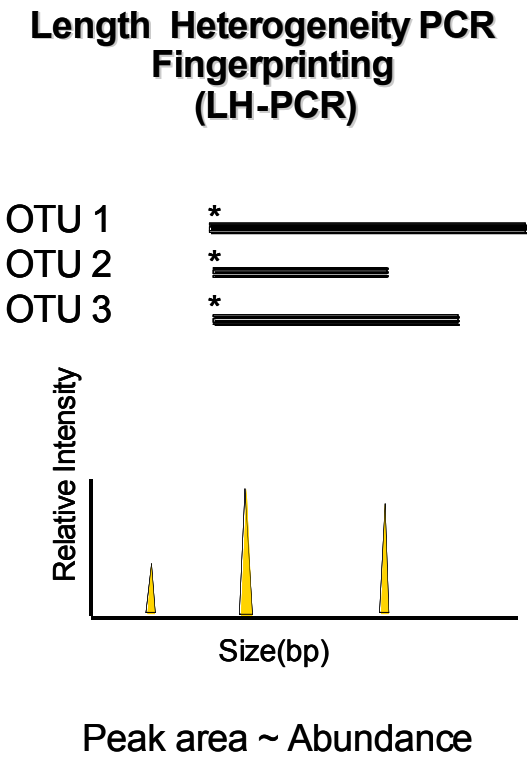


Figure 10. Location of primers on the SS rDNA molecule of *E. coli* and example output from the fingerprinting protocols used in this study.

organism might be present in a fingerprint of a whole genomic extract. However, we obtained *Aurantimonas coralicida* DNA from an isolate kindly provided by Dr. L. Richardson and determined its specific LH length and extracted genomic DNA from the range of bacterial isolates derived from the coral samples. This gave an empirically determined signature for an etiological agent of WP as well as signatures for the breadth of culturable bacteria.

Isolated colonies from the culturable bacterial community are being analyzed for their unique LH signatures and sequenced to aid in their identification and to compare with OTUs from community DNA extracts. This approach, with LSI and STX coral samples, has indicated that a very large proportion of the bacteria in coral tissues (approximately 70 % thus far) can be cultured on half strength marine agar.

For microbial analyses, after return to GMU, each Petri dish was photographed with a high-resolution digital camera to provide a permanent record of both abundance and diversity of the culturable bacterial community (Figure 11). The abundance of colonies has been determined and their diversity is being analyzed using Bioquant image analysis software. The use of high-resolution photography for this application will be very valuable for long-term comparison since the actual incubated plates degrade over time.

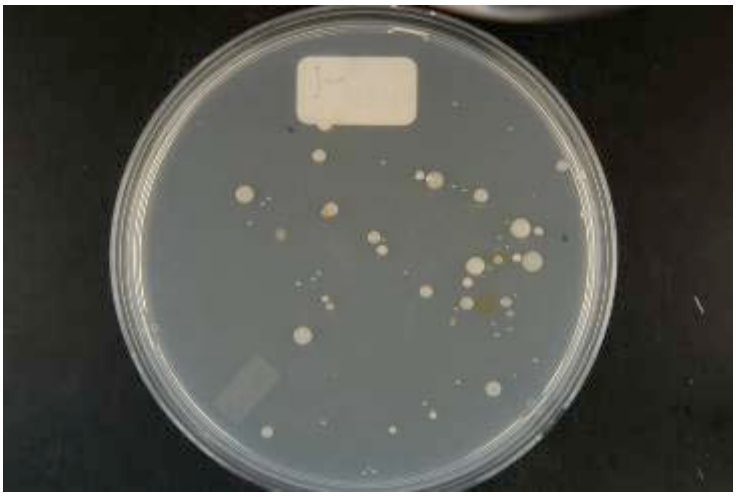


Figure 11. Typical bacterial colonies on a plate of one-half strength Marine Agar 2216.

RESULTS:

Culturable Bacteria

Bacterial colonies developed on all of the marine agar plates. There are very large, culturable bacterial communities associated with these coral tissues (Table 1). When all the coral tissues data were averaged a total of more than 1 x 10^8 CFU per gram of tissue were present (Table 1). There was substantial variability in bacterial abundance indicted by the wide standard

Table 1. Mean and standard deviation of culturable bacterial abundance in coral tissue samples plated on ½ strength Marine Agar 2216.

Mean Bacteria Abundance Flower Garden Banks National Marine Sanctuary May 2005	Total Culturable Bacteria Marine Agar 2216 (½ strength at ambient salinity)	
	Mean CFU/g coral tissue	Standard Deviation
All Coral Tissue Samples	1.12E+08	1.43E+08
Diseased Coral Tissue	1.46E+08	1.34E+08
Healthy Tissue on Diseased Colony	8.08E+07	1.08E+08
Healthy Tissue on Healthy Colony	9.85E+07	1.82E+08

deviation. The highest average bacterial abundances, almost 1.5 x 10^8 CFU/g, occurred in the white-disease affected coral samples from the healthy tissue-bare skeleton interface. Out previous investigation of bacterial abundance in coral tissues from *M. annularis* complex also indicated slightly higher culturable bacterial abundances in samples at the disease interface. Lowest abundances, but still more than 8 x 10^7 CFU/g, were on healthy tissue from diseased colonies. The difference among all three sample types was modest, and, given the wide standard deviations among sample pairs, are not statistically significant.

In two of the four samples pairs (EC301/306, *M. faviolata* and WC201/206. *M. franksii*) bacterial abundance along the disease interface was substantially greater than in healthy tissue samples (Figure 12), both from the diseased colony as well as the healthy control colony. In the other two pairs (EC4021/406, *M. faviolata* and WC101/106, *C. natans*) there was not much difference among all tissue types, and in the case of WC101/106 the highest abundances occurred in the healthy tissue on the apparently healthy colony. Perhaps the most striking result shown in Figure 12 is the large variation in culturable bacterial abundance in coral tissue on apparently healthy colonies with the highest value present on *C. natans* being an order of magnitude higher than on any of the healthy *Montasraea* tissue samples. Although the difference was small in one case, white-disease-affected colonies of *Montasraea*, but not *Colpophyllia*, all had higher abundances of bacteria along the disease margin than in healthy tissue.

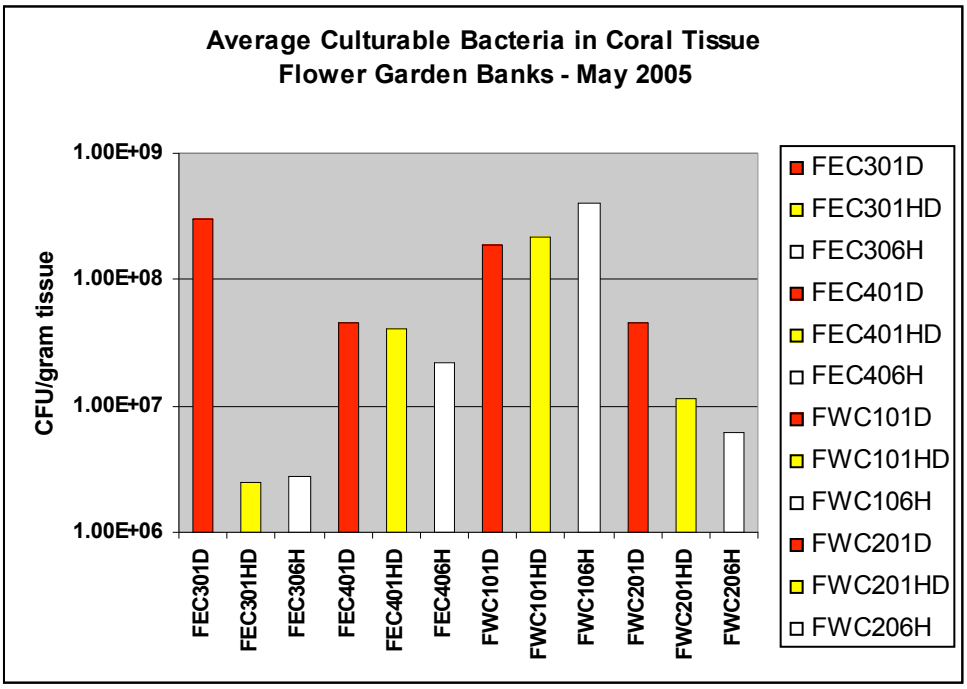


Figure 12. Average bacteria abundance among three samples collected on each pair of corals, one affected by white disease and one apparently healthy colony. Red – diseased interface, Yellow –healthy tissue on diseased colony, white – healthy tissue.

Culturable bacterial abundances in individual tissue cores are presented in Figure 13. In most cases the variation in abundance among triplicate tissue samples was rather small. However, in several cases they differed by more than an order of magnitude (e.g. EC306 and WC301D). Of course this result could be due to processing variation or sample size differences, however, samples were processed methodically and the trimmed samples were all very similar in

total volume. It seems likely that the differences reflect real variations in bacteria community composition. Nevertheless, it appears that white plague-like disease present at FGBNMS in May 2005 probably cannot be distinguished in tissue samples merely on the basis of total culturable bacterial abundances when plated on this nutrient medium. In the genus *Montastaea* it might be suggested that average abundance values greater than 1 x 10^8 CFU/g are associated with a diseased state, although this concept obviously requires more testing. In *C. natans* it appears that normal bacterial abundances, > 1 x 10^8 CFU/g, exceed those in healthy *Montastaea* tissue.

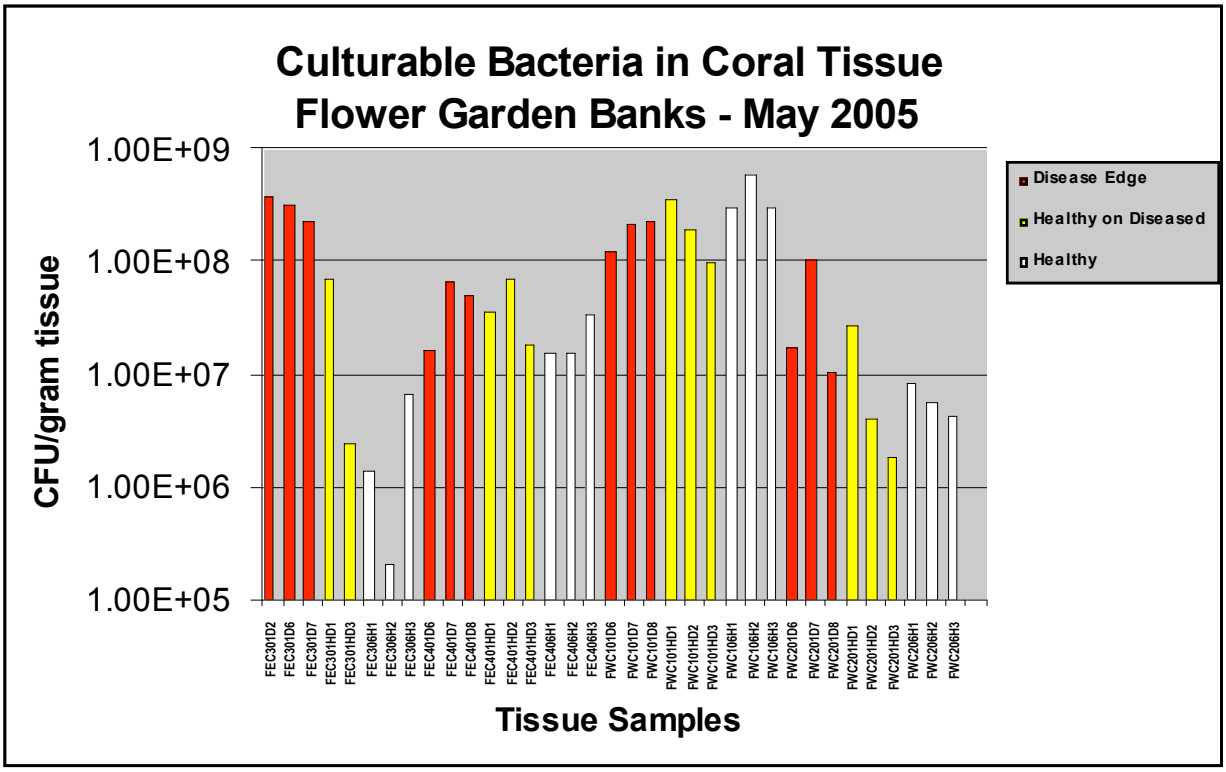


Figure 14. Bacterial abundance from each tissue of the triplicate tissue samples from four pairs of corals, one affected by white disease and one apparently healthy colony. Red – diseased interface, Yellow –healthy tissue on diseased colony, white – healthy tissue. EC301/306 and EC401/406 *M. faviolata*, WC101/106 *C. natans*, WC201/206 *M. franksii*.

We can compare the results from FGBNMS corals with our previous investigation of bacterial community composition in white-plague diseased and healthy *M. annularis* (complex) colonies. Overall bacterial abundance at FGBNMS was between the high value found on coral tissue from LSI (approximately 3 x 10^8 CFU/g) and the lower value from STX corals (approximately 4 x 10^7 CFU/ml). This relative relationship was consistent for all three tissue types from the three reef sites. The general trend of highest abundance being associated with the disease margin was also true for all three sample sets for the genus *Montastraea.*

One important experimental issue is that there was significant variation in culturable abundance among the different paired samples. This information is vital to efficiently dilute samples for plating. Theoretically it is possible to test many dilutions, but carrying large quantities of media to the field site makes this approach very difficult and expensive. We adjusted our dilutions during the sample trips based on early observations of colony development. Small-scale geographic difference, different locations in the same reef tract, seemed to have as much or more significance in variations in bacterial abundance than did health status of the coral colonies and individual samples.

Again for comparative purpose, culturable bacteria in the sediment at LSI and STX ranged from about 3×10^5 to 6×10^7 CFU/g. These are modest values for sediments. Sediment abundances in LSI sediments were about an order of magnitude greater than those at STX. Although, no sediment samples were collected at FGBNMS, it is instructive to note that the culturable bacterial community is more abundant, on a weight basis, on corals than in the sediment. It seems likely that this reflect the high nutrient availability on and in the coral tissue as compared with coarse, reef sediment.

From a morphological perspective no golden-yellow colonies, indicative of possible *A. coralicida*, developed on any of the nutrient plates even after seven days of incubation. Nevertheless, we are screening the isolates for LH signatures that match *A. coralicida*. particularly obvious in healthy coral tissue from these sites. However, the diseased tissue from STX contains significantly more OTUs than does the healthy tissue. All of these data indicate that a unique bacterial community characterizes WPII-diseased tissue at each site and that that community differs in composition and/or relative abundance from healthy tissue.

ADDITIONAL PERSONNEL (Students, Other scientists, technicians, etc.):

Geoffrey Cook, Master of Science student, Department of Environmental Science and Policy

Masi Sikaroodi, Doctoral student and Research Associate, Department of Molecular and Microbiology

Karen Santora, Master of Science student, Department of Environmental Science and Policy

GPS COORDINATES OF RESEARCH SITES:

Mooring Buoy Coordinates: East Bank

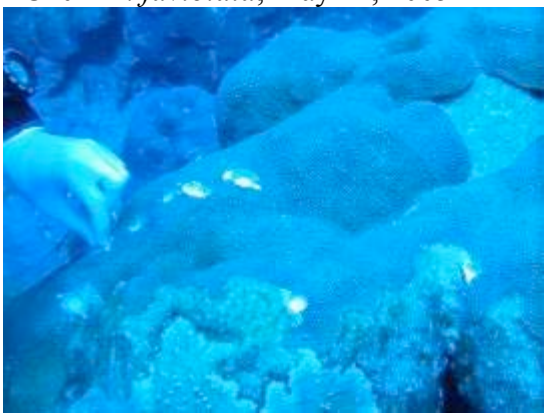
Position	Latitude	Longitude
3	27°54'27.0" N	93°35'57.4" W
4	27°54'33.0" N	93°35'59.7" W

Mooring Buoy Coordinates: West Bank

Position	Latitude	Longitude
1	27°52'35.1" N	93°48'54.1" W
2	27°52'31.3" N	93°48'51.3" W

Images of diseased coral colonies sampled during this program.

EC401 *M. faviolata*, May 24, 2005



WC201 *M. franksii*, May 26, 2005