BACTERIAL PROFILES IN HEALTHY AND MONTIPORA WHITE SYNDROME AFFECTED MONTIPORA CAPITATA MUCUS AND THE IDENTIFICATION OF POTENTIAL ETIOLOGIC AGENTS

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ABSTRACT

Montipora white syndrome (MWS) is a progressive tissue loss disease that affects Montipora capitata, a major reef building coral in Kaneohe Bay, Oahu. Chronic MWS manifests as focal to multifocal variably-sized areas of tissue loss, revealing an intact white skeleton bordered by normal appearing tissue. Acute MWS progresses faster than chronic and manifests as a large solitary area of tissue loss revealing intact white skeleton bordered by pale tissue that transitions into normally colored tissue. Culture-dependant methods were used to determine the bacterial community structure associated with healthy and MWS affected M. capitata mucus. Healthy mucus contained $5.59 \times 10^2$ CFU/ml of culturable bacteria that was predominantly Alteromonas and Streptomyces. MWS affected mucus samples had an average of 25.6 times higher bacterial load at $1.42 \times 10^4$ CFU/ml of mucus. The culturable bacterial community structure of mucus from diseased coral was primarily composed of Vibrio spp., in particular, V. harveyi. The second most abundant bacteria isolated from mucus was Pseudoalteromonas and Ruegeria in acute and chronic MWS, respectively. Based on these bacterial profiles, Alteromonas, V. harveyi, Pseudoalteromonas and Ruegeria isolates were selected for in vitro challenge experiments. Alteromonas was used as a negative bacterial control because it is commonly found in abundance in healthy M. capitata mucus. Preliminary challenge experiments eliminated V. harveyi and Ruegeria as etiologic agents. Though no tissue loss was observed, mucus after inoculation with Ruegeria had a slightly elevated CFU/ml. Fragments exposed to Pseudoalteromonas had varying responses to the challenge. Tissue loss, thinning and, alternatively, no apparent signs of deteriorating health were all observed after the challenge with Pseudoalteromonas. Overall, CFU/ml of mucus was increased post challenge with Pseudoalteromonas for all three observations. In future studies, additional challenge replicates should be conducted to further investigate the role of Pseudoalteromonas in coral pathogenesis.
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INTRODUCTION

Coral Disease

Coral reefs are highly diverse, interconnected ecosystems. Slight shifts in environmental and anthropogenic stressors can cause downstream effects that degrade the reef (Barber et al. 2001). Coral disease can devastate coral populations and restructure overall reef composition (Barber et al. 2001; Aronson & Precht 1997). Loss of live coral decreases structural complexity, which in turn decreases associated species diversity of reef communities (Graham et al. 2006). Disease can destroy the major reef-building corals and, as a result, the reef composition shifts to one dominated by algae and non reef-building corals (Aronson & Precht 1997; Barber et al. 2001; Dustan & Halas 1987; Edmunds 1991). In one case, geologic analysis showed that such a shift in reef structure had not taken place in the last 4,000 years (Richardson 1998). This provides evidence that these disease outbreaks are restructuring communities that had remained stable for millennia.

There has been an increase in marine diseases reported worldwide (Harvell et al. 1999; Ward & Lafferty 2004). Many new coral diseases were reported in the 1990s, such as: red band disease, yellow band disease, white pox and sea fan disease, (Richardson 1998) and new diseases are still being identified today. Several of these diseases have been linked to environmental stressors such as increased temperature, sewage input and run off, nutrients and sediment loading (Rosenberg & Ben-Haim 2002; Kaczmarsky et al. 2005; Sutherland et al. 2004). Questions have been posed as to what is causing this disruption in stability. Is the environment different? Are the corals becoming more susceptible to disease? What are the biotic and abiotic factors involved? What are the pathogens involved? Are the pathogens becoming more virulent? As these questions continue to be
answered, understanding of disease processes may have management implications that may contribute to the control of disease progression.

**Coral-Associated Bacteriology**

In an effort to characterize emerging diseases, coral-associated microbes have become an area of interest. Due to this increased scrutiny, the interactions between corals and microbes are beginning to be understood. The surface mucopolysaccharide layer is a balanced environment that supports bacterial growth (Sharon & Rosenberg 2008). It contains levels of oxygen, nitrogen, phosphorus, monosaccharides and amino acids that are sufficient to support bacterial growth at $>10^8$ cells/ml (Sharon & Rosenberg 2008; Klaus et al. 2007). Additions of oxygen, nitrogen and phosphorus increase the cell yield by up to six times (Sharon & Rosenberg 2008).

The coral environment has been shown to have distinct consortia of bacteria associated with both their mucus and tissue layers (Koren & Rosenberg 2006). These communities are distinct from one another and therefore are specific to these areas of the coral (Koren & Rosenberg 2006). Mucus-associated communities of bacteria are specific to the mucus environment as illustrated by the distinct community profiles compared to the surrounding seawater (Koren & Rosenberg 2006). These bacterial profiles are also, despite geographical distances, specific to their coral species (Rohwer et al. 2002; Rosenberg et al. 2007). Neighboring coral colonies of different species harbor differentially structured bacterial profiles; whereas, the same coral species, separated geographically, have similar bacterial communities (Rohwer et al. 2002; Rosenberg et al. 2007). These associations,
though species specific, may be affected by external factors such as depth in the water column and pollution (Klaus et al. 2007).

The mucus-associated bacterial environment plays a role in coral resistance to infection. The mucus itself regulates the consortium found within it by down-regulating growth of some bacterial species as well as favoring growth of other bacterial types, such as Photobacterium spp. and Halomonas spp. which produce antimicrobials (Ritchie 2006). Although the mechanism was not investigated, this was determined by type and abundance of bacteria cultured on mucus treated and untreated media (Ritchie 2006). Extrinsic to mucus shaping the healthy-associated bacterial community, members of the bacterial community also help regulate the resident population. Some bacteria associated with healthy mucus produce antimicrobials that prevent colonization by potentially invading or opportunistic species of bacteria (Ritchie 2006). In combination, the coral mucus and mutualistic bacteria provide an environment that encourages the growth of some bacteria and prevents the growth of others (Ritchie 2006).

Within the bacterial community associated with coral mucus there are also complex bacteria-bacteria interactions competing against one another. Production of antimicrobials can help bacteria secure their space within the community and increase access to available nutrients (Rao et al. 2005). Brinkhoff et al. (2004) showed Roseobacter gallaeciensis, isolated from a water sample from the Wadden Sea, produces an antibiotic, tropodithietic acid, which inhibits the growth of strains of Flavobacteria, Actinobacteria and some Alphaproteobacteria. Gammaproteobacteria are nearly unaffected by the compound and are found to be a very resilient group against antagonistic products (Brinkhoff et al. 2004). However, there are variations in susceptibility within taxa. Bruhn et al. (2005) showed the
presence of thiotropocin or tropodithietic acid inhibits growth of two Gammaproteobacteria, *Vibrio anguillarum* and *V. splendidus*. *Pseudoalteromonas* spp. are rapid growing and competitive fouling species and therefore produce a wide range of anti-fouling products (Rao et al. 2005). Illustrating the dynamic inter-genera interactions, Rao et al. (2005) showed *P. tunicata* out-competes *Alteromonas* sp. in the production of a biofilm but is, in return, outcompeted by *Roseobacter gallaeciensis*. However, in preestablished biofilms, Rao et al. also showed *P. tunicata* is able to remove competitors after 120 hours due to expression of the antibacterial protein, AlpP (2005). *Alteromonas* spp. produce a wide variety of inhibitory products. *Alteromonas* inhibits growth of *P. tunicata* and *R. gallaeciensis* (Rao et al. 2005). An *Alteromonas* species was also shown to produce a broad-spectrum bacteriostatic product, PQ (2-n-pentyl-4-quinolinol), which inhibits growth of some alphaproteobacteria, *Cellulophaga fucicola, Pseudoalteromonas* spp. and *Vibrio* spp. (Long et al. 2003; Baraja et al. 1989). These complex interactions appear to add to the stability of healthy corals by inhibiting the overgrowth of specific bacteria.

**Known Etiologies of Coral Disease**

Over 20 coral diseases have been described in the past 40 years and the number continues to rise (Rosenberg et al. 2007). Despite the continual emergence of new coral diseases and our increasing knowledge of coral-microbial interactions, only a small number of etiologies have been characterized. Only six of these diseases have etiologies characterized using Koch’s postulates; five of which are bacterial infections (Rosenberg et al. 2007). Diseases with known etiologies are: black band disease (BBD), white pox,
Aspergillosis, white plague (WPL), *Porites* trematodiasis, yellow blotch disease (YBD) and bacterial bleaching caused by *Vibrio* spp. (Rosenberg *et al.* 2007).

Black band disease was first described in Belize in 1973 (Antonius 1973). It has since been found to affect various scleractinian species, fire corals and gorgonians in reefs throughout the Indo-Pacific and Caribbean (Bruckner *et al.* 1997; Kuta & Richardson 1996; Littler & Littler 1996). It is characterized by a microbial mat that forms a black band which separates the living tissue from areas of recent tissue loss. Data surrounding the pathogen of BBD is contradictory. Results of bacterial consortium analyses reveal unique results for different coral colonies (Cooney *et al.* 2002; Sekar *et al.* 2006). The dominant microorganisms in the microbial mat include a sulfide-oxidizing bacteria, *Beggiatoa*, a sulfate-reducing bacteria, *Desulfovibrio*, and cyanobacteria, including *Phormidium corallyticum* (Garrett & Ducklow 1975; Ducklow & Mitchell 1979; Richardson 1996; Cooney *et al.* 2002). This variation of members and dominant taxa makes assigning a primary pathogen difficult though it is widely believed to be a consortium of bacteria. Although the consortium is diverse, pathogenesis is consistently due to the production of elevated bacterial sulphide which kills the coral tissue (Rosenberg *et al.* 2007; Sutherland *et al.* 2004). A vector, the butterflyfish *Chaetodon capistratus*, may be involved in increasing the rate of transmission of BBD. Feeding on infected and non-infected colonies as well as defecation on colonies may lead to the increased transmission of the bacteria responsible for BBD (Aeby & Santavy 2006). This illustrates the importance of external factors in the infectivity and transmission of BBD.

White pox was first documented in 1996 off the coast of Key West, Florida (Holden 1996) and has since been observed in reefs throughout the Caribbean (Porter *et al.* 2001). It
is a tissue loss disease that affects only one coral species, Acropora palmata. It is characterized by irregularly shaped, variably sized areas of tissue loss throughout the colony. Although originally termed a “pox”, disease nomenclature state associated with a viral etiology, Serratia marcescens was identified as the causative agent of white pox by Patterson et al. (2002). White pox is now more formally known as acroporid serratiosis and is the first known coral pathogen that is also a fecal enteric bacterium. This suggests a possible association of acroporid serratiosis with pollution of fecal origin (Patterson et al. 2002).

Aspergillosis of sea fans, Gorgonia ventalina, was first documented in 1995 and has caused mass mortalities in the Caribbean (Nagelkerken et al. 1997; Kim et al. 2006). It is characterized by purpling of the tissue and tissue loss and is caused by the fungus Aspergillus sydowii (Geiser et al. 1998; Smith et al. 1996). Aspergillus sydowii is found in both terrestrial and aquatic systems. Initial studies using terrestrial strains of A. sydowii were inoculated onto G. ventalina fragments for a comparison with the marine strains of A. sydowii. The terrestrial strains did not induce a disease state. Therefore, it is hypothesized that these marine isolates cultured from diseased sea fans must possess pathogenic factors that the terrestrial strains do not possess (Geiser et al. 1998; Sutherland et al. 2004). This emphasizes the fact that more research is needed to understand virulence factors.

White plague is a rapid tissue loss disease that affects Caribbean corals and is divided into WPL I, WPL II and WPL III with increasing rates of tissue loss, respectively (Richardson et al. 1998; Richardson et al. 2001). The causative agent of WPL II is a novel genera and species of bacteria, Aurantimonas coralicida. Aurantimonas coralicida is a Gram-negative, rod-shaped alphaproteobacteria with 16S rDNA sequence similarity not
higher than 92% to other members of the family (Denner et al. 2003). Modes of infection and transmission are currently unknown (Denner et al. 2003; Sutherland et al. 2004). Another bacterium, *Thalassomonas loyana*, was cultured from WPL-like infected *Favia favus* in the Red Sea. This bacterium induces white plague-like signs in aquaria; however, re-isolation of the strain from additional studies was unsuccessful (Barash et al. 2005; Thompson et al. 2006; Rosenberg et al. 2007). Based on current knowledge, the wide geographic and host range of WPL as well as similar disease signs between types I, II and III does not support the diagnosis of a single etiology for all WPL diseases (Lesser et al. 2007).

*Porites* trematodiasis is caused by the infection of *Porites* spp. by the digenetic trematode, *Podocotyloides stenometra* Pritchard (Aeby 1998). *Porites* trematodiasis affects *Porites* spp. throughout the Pacific (Chen & Wong 1974; Bray & Cribb 1989). *Porites* spp. serve as the second intermediate host for the trematode and infection is characterized by the appearance of pink nodules on the coral colony (Aeby 1998). The final host of *Podocotyloides stenometra* is corallivorous fish; one of which is the butterflyfish, *Chaetodon multicinctus*, which preferentially feeds on infected polyps (Aeby 2002). Parasitized *Porites* colonies experience a reduction in growth rate up to 50% (Aeby 1992). Predation by corallivorous fish decreases the number of cysts present on the colony (Aeby 1992). Transfer of the parasite to its final host and senescence of metacercaria not transferred by fish bite allows *P. compressa* colonies to return to normal growth rates (Aeby 1992). Preferential feeding of corallivorous fish on infected polyps increases the rate of parasitic transmission to the final host as well as facilitates removal of infected polyps from the colony (Aeby 1992).
In contrast to diseases with a wide range of specific etiologies, Vibriosis causes a number of coral diseases with different disease signs. *Vibrio* species can be pathogenic as well as opportunistic colonizers (Thompson *et al.* 2004; Ritchie 2006; Dinsdale *et al.* 2008; Rosenberg & Ben-Haim 2002; Breitbart *et al.* 2005). Since some Vibriosis is associated with elevated temperatures, this shift to a *Vibrio* dominated community may be attributed to a temperature-dependant up-regulation of virulence factors (Ben-Haim *et al.* 2003; Vattakaven *et al.* 2006; Banin *et al.* 2003). Up-regulation of virulence factors, i.e., protective enzymes, may allow *Vibrio* spp. to out compete the normal mucus flora of healthy coral mucus (Ritchie 2006). The production of superoxide dismutase and catalase allow survival and protection of *Vibrio* spp. in the high oxygen environment of the coral host (Munn *et al.* 2008). Vibrios also have type II and III secretion systems and quorum sensing (Evans *et al.* 2008). These systems play a role in pathogenicity through the regulation of expression and export of virulence factors, as well as the export of proteins and inhibitory secondary metabolites (Evans *et al.* 2008, Thompson *et al.* 2004). These compounds, which inhibit Alphaproteobacteria and *Alteromonas* spp., are more likely to be produced upon attachment to a surface (Long & Azam 2001). *Vibrio* spp. form biofilms on a variety of marine organisms and a study of *V. cholerae* shows surface attachment after only 15 minutes (Thompson *et al.* 2004; Watnick & Kolter 1999). This suggests that Vibrios are able to attach to available surfaces quickly and maintain their niche once they have secured attachment.

The ability of *Vibrio* spp. to quickly colonize and produce virulence factors may aid in their ability to opportunistically dominate diseased coral bacterial communities. They have been characterized as pathogens in two coral diseases: YBD and bacterial bleaching of
coral (Cervino et al. 2004; Ben-Haim et al. 2003; Kushmaro et al. 1997). Some of the virulence factors produced by these pathogens are induced by increased temperatures which correlates the increasing virulence of the pathogen with the warming environment (Ben-Haim et al. 2003; Vittakaven et al. 2006).

Yellow blotch disease affects several scleractinian coral species, but is most commonly linked to Montastraea spp. YBD manifests as a pale-yellow spot on the coral and progresses into a larger band as the disease develops (Cervino et al. 2004). The disease state is induced by four distinct Vibrio spp. When inoculated in combination, the disease signs appear quicker and more closely represent field signs of YBD than when inoculated separately (Cervino et al. 2004). Vibrios seem to target the symbiotic zooxanthellae, and leave the coral tissue alive. When the temperature increases, YBD fragments do not expel their zooxanthellae, as do the healthy fragments, because the zooxanthellae are lysed in the gastroderm of the coral host (Sutherland et al. 2004; Cervino et al. 2004). After YBD infection, cytoplasmic and organelle integrity is compromised and chlorophyll a pigments are drastically decreased in the zooxanthellae while the host tissue remains intact (Cervino et al. 2004). This targeting of the zooxanthellae as opposed to the host tissue suggests YBD is a disease affecting the zooxanthellae compartment of the coral symbiont (Cervino et al. 2004)

Bleaching and lysis of Pocillopora damicornis tissue by V. corallilyticus is a temperature-dependant process (Ben-Haim et al. 2003; Ben-Haim et al. 2003). Challenge experiments show bleaching of P. damicornis at 24°C. This bleaching process decreases the concentration of zooxanthellae to less than 12% of the concentration in healthy fragments (Ben-Haim et al. 2003). At an increased temperature range, 27-29°C, V.
corallililyticus lyses >50% of coral tissue within two weeks. In this temperature range, levels of extracellular protease activity increase in V. corallililyticus, increasing bacterial-induced lysis (Ben-Haim et al. 2003). Unlike bleaching by V. shiloi, it is unknown whether increased temperature increases susceptibility in P. damicornis or increases the virulence of V. corallililyticus (Ben-Haim et al. 2003; Ben-Haim et al. 2003).

Bleaching of Oculina patagonica by V. shiloi is the best described of the infectious coral diseases. The steps to pathogenesis by V. shiloi are well characterized. Initially, V. shiloi moves toward the mucus of O. patagonica by positive chemotaxis and adheres to the coral surface using β-D-galactopyranoside-containing receptors located on the surface of the mucus layer. V. shiloi penetrates into the epidermal layer of the coral and enters a viable but not culturable state (Banin et al. 2001; Rosenberg et al. 2007; Vattakaven et al. 2006). Intracellular replication to cell densities above $10^8$ cells/cm$^3$ of coral tissue is made possible by the production of secreted superoxide dismutase. This virulence factor is critical for V. shiloi survival in the coral tissue due to high levels of oxygen radicals produced by the zooxanthellae (Banin et al. 2003). A proline-rich peptide is produced which binds to zooxanthellae, inhibits photosynthesis and lyses the cells (Banin et al. 2001).

**Bacterial Challenge Methods**

Determining the etiologies of coral diseases is a relatively new focus of research and published methods are highly variable (Denner et al. 2003; Sharon & Rosenberg 2008; Banin et al. 2003; Patterson et al. 2002). The need for standardization and optimization of methods in this field is necessary for the future of this expanding area of research.
Challenge methods for coral disease are problematic because it is often difficult to ensure that the same disease has been reproduced in the laboratory and impossible to reproduce the same conditions in the laboratory as are present in the field (Lesser et al. 2007). Fulfilling Koch’s postulates for coral disease requires the identification of coral’s normal flora and water-associated bacteria as background to bacterial communities associated with diseased corals (Ritchie et al. 2001). Some researchers suggest Koch’s postulates are not ideal for coral disease investigations because they require that the pathogen is culturable, and that healthy coral are placed in artificial aquarium environments and observed for disease signs resembling those in the field (Lesser et al. 2007, Ritchie et al. 2001). They also do not take into account tank effects that may also initiate disease signs. These postulates do not account for opportunistic pathogens that may arise after environmental stress (Lesser et al. 2007). Currently, the methods being used to satisfy these postulates for coral disease are inconsistent (Denner et al. 2003; Sharon & Rosenberg 2008; Banin et al. 2003; Patterson et al. 2002). Major variations include bacterial isolation methods, inoculation methods and aquarium challenge protocols.

Methods for sampling coral-associated bacteria include the use of coral crushate, (Ben-Haim et al. 2003; Denner et al. 2003) syringe samples of mucus collected in the field (Ritchie 2006; Patterson et al. 2002) and mucus removed after transport to the lab (Sharon & Rosenberg 2008). Crushate allows for culture of the specific bacterial communities associated with the mucus as well as the tissue. However, the crushate slurry dilutes the sample drastically and cellular lysates may kill or decrease the number of individual bacterial types. Syringe collection in the field minimizes coral damage, but increases the probability of the surrounding water, and water-associated bacteria, entering the syringe
with the mucus sample. Bringing fragments back to the lab prevents water contribution, but allows time for mucus sloughing, resulting in the loss of associated bacteria.

The maintenance protocols for coral fragments in wet lab conditions prior to challenge experiments are also highly variable. In white pox challenge experiments, fragments of *A. palmata* were acclimated in a flow-through system for three days prior to inoculation (Patterson *et al.* 2002). Fragments were allowed to acclimate for 15 days before inoculation with *V. shiloi* and a variable time over two weeks for *V. coralliliiyticus* experiments (Banin *et al.* 2003; Ben-Haim *et al.* 2003). Flow-through systems may not provide fragments with similar light conditions or water motion as they had in the field. The more time fragments are left in flow-through systems to acclimate, the more time they are exposed to an artificial environment that could bring predators and an influx of bacteria associated with biofilms on inflow hoses and pipes.

Bacterial inocula preparation for challenge experiments also varies widely. Growth of bacteria for use in challenge experiments ranges from overnight cultures diluted to $10^9$ to the use of cultures grown for a specified time period (Banin *et al.* 2003; Ben-Haim *et al.* 2003). This creates variation in growth phase and cell density of the bacteria for use in challenge experiments. Inoculation methods are broad and only consistent within research groups. *Vibrio shiloi*, at a concentration of $10^9$, was inoculated into aquaria filled with two liters of seawater (Banin *et al.* 2003). *Vibrio coralliliiyticus*, at a concentration of $10^7$, was inoculated onto completely exposed *P. damicornis* fragments and left exposed for one minute before placing in aquaria (Ben-Haim *et al.* 2003). *Serratia marcescens* was mixed with calcium carbonate sediment for bacterial absorbance by the sediment before
inoculation of the sediment mixture directly onto the coral in white pox challenge experiments (Patterson et al. 2002).

Not all of these methods may be standardized across the field, but optimization for specific diseases and coral species under study is required. Accounting for growth phase of the inoculum as well as possible sources of contamination will help standardize methods. Inoculation method, acclimation time and length of experimental run time should be optimized for specific coral species and disease. As more challenge protocols are developed, movement toward consistency needs to be made for coral disease researchers to develop a unified approach for investigations.

**Montipora White Syndrome**

*Montipora* White Syndrome (MWS) is a progressive tissue loss disease that affects *Montipora capitata*, a major reef building coral in Kaneohe Bay. MWS has been observed in the Northwestern and Main Hawaiian Islands (Aeby 2004). It was first documented in Kaneohe Bay, Oahu in 2004 where levels of the disease were found to be higher than elsewhere in Hawaii. In a comparative study conducted in Kaneohe Bay, this disease has no apparent seasonality; however, prevalence is increased on human impacted reefs (Aeby 2007 HCRI-RP Final Report). Preliminary lab experiments were completed to test transmissibility of MWS by direct and indirect means. Two healthy fragments were placed in an aquarium with a MWS affected fragment. One healthy fragment was touching the diseased fragment to test direct transmission. The second healthy fragment was placed in the same aquaria but not touching either of the other two fragments to test indirect transmission. These experiments showed MWS to be transmissible through direct contact.
100% of the time as well as through indirect transmission, though only ~50% of the time. Transmission in this manner suggests MWS is caused by an infectious agent.

Tissue loss of MWS progresses at a rate of 3.1% of the affected colony per month. Thirty percent of tagged colonies in Kaneohe Bay experienced ≥90% tissue loss after one year. Grossly, two disease states were observed. Chronic MWS is more common and manifests as focal to multifocal variably-sized areas of tissue loss, revealing an intact white skeleton bordered directly by normal appearing tissue. Acute MWS progresses faster than chronic and manifests as a large solitary area of tissue loss revealing intact white skeleton bordered by pale tissue that transitions into normally colored tissue (Aeby 2007 HCRI-RP Final Report).

Most of the coral pathogens identified to date are culturable bacteria (as reviewed in Rosenberg et al. 2007). Whether this is due to the biomedical approach used by many investigations or the difficulty experienced when trying to factor in abiotic factors is a point for future investigations. However, culture dependant microbial methods are a good starting point for characterizing MWS. Identifying mucus-associated bacterial communities from healthy as well as acute and chronic MWS affected M. capitata will allow for identification of shifts in the bacterial community and selection of potential etiologic agents. Culture based methods will allow potential pathogens to be used in subsequent challenge experiments. Due to the variety of methods being employed in challenge experiments, aspects of the experiments will be scrutinized to optimize the system for M. capitata and MWS. Should the results for this approach show culturable bacteria do not cause MWS, there is still merit in performing these experiments to rule out culturable bacteria as the etiological agent(s) of MWS.
Project Objectives

- Characterize the culturable mucus-associated bacterial community structure of healthy, acute and chronic MWS affected *M. capitata*
- Optimize *in vitro* challenge protocols for MWS
- Determine whether culturable bacteria occurring in increased numbers in MWS affected corals can be used to fulfill Koch’s postulates
METHODS

Coral Collection

_Montipora capitata_ fragments were collected from Kaneohe Bay, Oahu, Hawaii. Five healthy colonies, five acute MWS affected colonies and five chronic MWS affected colonies were selected along a single fringing reef near the Hawaii Institute of Marine Biology on Coconut Island. Colonies were tagged and photographed using an Olympus C-5050 digital camera and underwater housing (Olympus America Inc., Center Valley PA). Colony depth, size and the percentage of the colony with tissue was recorded for each of the selected _M. capitata_ colonies. For consistency, all colonies selected shared the same orange, branching morphology. Coral fragments were transported immediately back to the lab in plastic bags without water to minimize the amount of mucus sloughed off during transfer. Mucus was collected from fragments using a P-1000 pipetter (Rainin, Oakland CA). For MWS affected fragments, mucus was collected from the disease progression front.

Bacterial Cultures

Mucus was plated on Glycerol Artificial Sea Water (GASW) (Smith & Hayasaka 1982) and Thiosulfate-Citrate-Bile-Sucrose (TCBS) (VWR, West Chester PA) (Kobayashi et al. 1963) using 60 x 15mm culture plates. Mucus was inoculated in triplicate undiluted and at 10-fold serial dilutions (10^{-1} - 10^{-4}). Cultures were incubated at 25°C for 48 hours. Serial dilutions were done in 0.22um filtered sea water (FSW). Colony forming units per ml (CFU/ml) of mucus was calculated based on average numbers of colonies counted on GASW media for the dilution that had 50-200 colonies overall. Colony counts on TCBS
media were used as a tool for rapid calculation of the proportion of putative *Vibrio* spp relative to CFU/ml on paired GASW cultures. One replicate GASW plate per fragment was selected for comprehensive sequence analysis. Each colony from the representative plate was selected and patched onto a GASW plate. After 48 hours at 25°C, bacterial isolates from patches were used to inoculate GASW liquid media. Cultures were grown in a shaker at 25°C and 230 rpm until approximately log phase (OD$_{600}$ = 0.10). Sixty percent glycerol was added to the culture to make a 20% glycerol solution (v/v) that was stored in triplicate at -80°C for later use and archival purposes.

*Molecular Methods*

Bacterial DNA was extracted from patch cultures using either the MoBio Microbial or Soil Extraction kit (MoBio, Carlsbad CA). The extraction protocol was followed as per the manufacturer’s instructions with minor modifications. The lysis step was modified by increasing the incubation period to 15-20 minutes with two rounds of vortexing (2-3 minutes each) dispersed evenly during the 15-20 minute period. 16S rDNA was amplified using universal primers 8F and 1513R and following the PCR protocol as described in Sorokin *et al.* (2001) (Appendix 1). PCR products were run on a 1% agarose gel to visualize amplification and verify product size of approximately 1,505 base pairs. Visualization of PCR products was facilitated using a 1:100 dilution of Gel Star (Cambrex Bio Science, Rockland ME). PCR products were purified using ExoSAP (Roche Diagnostics, Mannheim, Germany). Sequences were analyzed using 4 Peaks (A. Griedspoor & T. Groothius, mekentosj.com) and the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1997).
**Growth Curves**

Based on abundance in representation in the sequence data a healthy-associated bacterium and potential pathogens were selected for challenge experiments. This healthy-associated bacterial isolate was included as a negative bacterial control. Cultures were grown in GASW liquid media overnight and used as inoculum for growth curves at 1ul per ml of media. Growth curves were completed in GASW and FSW at 25°C shaking at 230 rpm. Optical densities were read every hour using a NanoDrop spectrophotometer at 280 and 600nm (OD$_{280}$ and OD$_{600}$) until early stationary phase was reached (leveling off of growth rate for two time points). Results were graphed and statistics calculated using Excel (Microsoft Office Version 11.3.6).

**Bacterial Challenge Experiments**

*Montipora capitata* fragments were collected off the east side of Coconut Island, Kaneohe Bay, Oahu, Hawaii. Selected colonies were tagged and photographed prior to collection as previously described. Fragments were transported in plastic bags full of water to minimize crushing and abrasions caused by other fragments. Sufficient fragments were collected from each colony to provide one fragment per tank, one for mucus collection and extras to keep in the water table for the duration of the experiment. Fragments were allowed to recover in a flow through water table for 3-4 days. Two sets of fragments (two separate coral colonies) were run paired, i.e. one freshly collected (3-4 days) and one healed, for two weeks in the water table.
Preliminary experiments optimizing challenge methods were done as part of the 2007 HCRI funded MWS experiments by an undergraduate assistant (Megan Ross ZOOL 499 research paper). Air hoses and nuts (to weigh down air hoses) were autoclaved for sterility. Tanks, egg crate stands, zip ties and lids were sterilized using a 10% bleach solution, neutralized using a 10% sodium thiosulfate solution and rinsed copiously in FSW to remove any residual chemicals. Aquaria were placed in secondary containment to prevent the spread of bacteria in the case of leaks, overflow or aquaria breakage. Tanks were filled to 3-4" above the egg crate and two fragments (one from each colony) were placed onto the egg crate.

Bacteria selected for inoculation were grown in GASW and monitored for growth phase. Once bacteria reached mid log phase, as identified in the previous section, cultures were pelleted by centrifugation and the supernatant was removed. The pellet was resuspended in an equivalent volume of FSW. Cultures were pelleted again and the supernatant was discarded to remove any remaining media. Pellets were again resuspended to volume in FSW. One ml of each suspension was removed for serial dilutions. Dilutions $10^4$-$10^6$ were plated in triplicate on GASW to calculate CFU/ml of inoculum. Ten ml of culture was inoculated into aquaria directly above each coral fragment. After two hours had lapsed, the water level in the aquaria was filled to approximately three inches below the top with FSW and the air tubes were connected to provide water circulation.

Mucus was collected from one fragment from each colony and serial dilutions were plated in triplicate on GASW and TCBS to calculate starting CFU/ml of coral mucus prior to bacterial inoculation. Fragments were photographed every four days post inoculation to monitor healthy appearance during the experiment. Water changes were done every four
days by vacuum siphoning 2L from the tank using a sterile 25ml pipette and refilled using fresh FSW. The duration of the experiment was three weeks. During this time, the extra fragments collected were left in the water table to provide a water table control. At the end of the three weeks, the water, aquaria and coral fragments were sterilized using a 10% bleach solution. Mucus was collected from all fragments in challenge run #2 and serial dilutions were plated in triplicate on GASW and TCBS for comparison to the initial CFU/ml. Mucus from challenge runs #3-5 was only collected if tissue loss was present.
RESULTS

Colony Tagging

Healthy colonies were identified as H1-5 (Fig. 1). Colony location ranged from 2 to 5 feet depth in the water column with colony size ranging from 1.1 to 4 feet in diameter (Table 1). Acute MWS colonies were identified as A1-5 (Fig. 2). Colony location ranged from 3 to 6 feet deep in the water column with colony size ranging from 1.5 to 3 feet in diameter (Table 1). At the time of collection, acute colonies had tissue loss ranging from 10 to 80% of the colony (Table 1). Chronic MWS colonies were identified as C1-5 (Fig. 3). Colony depth ranged from 2 to 4 feet with colony size ranging from 0.66 to 2 feet in diameter (Table 1). At the time of collection, chronic colonies had tissue loss ranging from 10 to 70% of the colony (Table 1).

Figure 1. Colonies sampled for (A) Healthy 1, (B) Healthy 2, (C) Healthy 3, (D) Healthy 4, (E) Healthy 5 fragments.
Figure 2. Colonies sampled for (A) Acute 1, (B) Acute 2, (C) Acute 3, (D) Acute 4 and (E) Acute 5 fragments exhibited signs of acute MWS.

Figure 3. Colonies sampled for (A) Chronic 1, (B) Chronic 2, (C) Chronic 3, (D) Chronic 4 and (E) Chronic 5 fragments exhibited signs of chronic MWS.
Table 1. Physical parameters of coral colonies where fragments were collected. Colony depth, size, percentage of colony with tissue loss and disease type are recorded.

<table>
<thead>
<tr>
<th>Colony #</th>
<th>Collection Depth (Feet)</th>
<th>Diameter (Feet)</th>
<th>% Tissue Loss</th>
<th>MWS type</th>
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<td>3-4</td>
<td>1.1</td>
<td>NA</td>
<td>NA</td>
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<td>4</td>
<td>NA</td>
<td>NA</td>
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<td>H4</td>
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<td>A1</td>
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<td>1.5</td>
<td>80</td>
<td>Acute</td>
</tr>
<tr>
<td>A2</td>
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<td>2</td>
<td>10-20</td>
<td>Acute</td>
</tr>
<tr>
<td>A3</td>
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</tr>
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<td>A5</td>
<td>5-6</td>
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</tr>
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<td>C1</td>
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<td>Chronic</td>
</tr>
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<td>70</td>
<td>Chronic</td>
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Table 2. Total CFU/ml calculated from serial dilutions plated on GASW and *Vibrio*

CFU/ml calculated from serial dilutions plated on TCBS. H=Healthy, A=Acute,
C=Chronic, W=Water

<table>
<thead>
<tr>
<th>Coral Colony Number</th>
<th>CFU/ml (GASW)</th>
<th>CFU/ml (TCBS)</th>
<th>Proportion of <em>Vibrio</em> spp. (%)</th>
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<tr>
<td>H1</td>
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<td>140</td>
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<td>0.0</td>
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<td><strong>17</strong></td>
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<tr>
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<td><strong>7</strong></td>
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</tr>
<tr>
<td>Standard Deviation</td>
<td>515</td>
<td>11</td>
<td>1.1</td>
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</table>

* = Calculated without H1
**Colony Count Data**

Serial dilutions plated on GASW and TCBS media were used to calculate CFU/ml of bacteria from coral mucus. Healthy *M. capitata* fragments had an average of $5.59 \times 10^2 \pm 5.35 \times 10^2$ CFU/ml of mucus (mean ± s.d., n=9) (Table 2, Fig. 4). Because H1 was thought to be an outlier, five additional healthy fragments were collected and plated to determine CFU/ml to increase the number of replicates. Diseased fragments (acute and chronic combined) had an average of $1.42 \times 10^4 \pm 8.43 \times 10^3$ CFU/ml; 25.6 times higher than healthy fragments (mean ± s.d., n=10) (Table 2, Fig. 4). Comparisons on TCBS media revealed a 17.7 times higher proportion of putative *Vibrio* spp. in mucus from diseased coral compared with that of mucus from apparently healthy coral. Healthy fragments had 1.7% putative *Vibrio* spp. (Table 2, Fig. 5). Diseased fragments had 30.1% putative *Vibrio* spp. (Table 2, Fig. 5). One healthy fragment had an unusually high CFU/ml on TCBS plates, 26.9%, and was identified as an outlier (Table 2). As an outlier, this fragment was not included in subsequent analyses in this study. Mucus from acute and chronic MWS yielded similar CFU/ml at $1.49 \times 10^4$ and $1.36 \times 10^4$, respectively (Fig. 6). The proportion of putative *Vibrio* spp. was also similar between acute and chronic at 32.2% and 28.3%, respectively (Fig. 7). Water sample controls had an average of $5.93 \times 10^2 \pm 5.15 \times 10^2$ CFU/ml, similar to that of healthy mucus (mean ± s.d., n=10) (Table 2, Fig.4). Comparisons on TCBS media indicated that water samples had 0.6% putative *Vibrio* spp.

**Sequence Analysis**

All colonies from one representative plate each for healthy, MWS and water control isolates were sequenced. Sixty-seven colonies were selected from healthy GASW plates.
Eight-hundred colonies were selected from MWS GASW plates. Twenty-eight colonies were selected from water GASW plates. BLAST analysis of the 16S rDNA sequences allowed identification of individual bacteria cultured from healthy and diseased coral mucus. *Alteromonas* spp. (32%) and *Streptomyces* spp. (28%) were the majority of the total bacterial abundance cultured from healthy mucus (Fig. 8).

Mucus from MWS corals was dominated by *Vibrio* spp. (Fig. 9, Fig. 10). Bacteria cultured from acute MWS mucus were predominantly *Vibrio* spp. (70%) with the next highest proportion being *Pseudoalteromonas* spp. (10%) (Fig. 9). Approximately one third of the bacteria cultured from chronic MWS mucus were *Vibrio* spp. (66%) (Fig. 10). The next most dominant genera was *Ruegeria* spp. at 10% of the bacteria sequenced (Fig. 10). *Vibrio harveyi* was the dominant species among the *Vibrio* genera cultured from diseased mucus at 19% of the total (Fig. 11). Forty percent of the *Vibrio* spp. had 16S fragments that only BLAST identified to the genus level (Fig. 11). Bacteria cultured from the collected water sample were primarily *Neptunomonas* spp. (21%), *Pseudoalteromonas* spp. (16%) and *Streptomyces* spp. (16%) (Fig. 12).

Sequence data shows the majority of bacteria cultured from all environments were representatives of the Gammaproteobacteria. Water-associated bacterial communities were primarily made up of Actinobacteria (14%), Alphaproteobacteria (18%) and Gammaproteobacteria (60%) (Fig. 13). Actinobacteria, Alphaproteobacteria and Gammaproteobacteria contributed 27%, 12% and 59%, respectively to the community profile of healthy *M. capitata* mucus (Fig. 14). MWS affected *M. capitata* mucus was dominated by Gammaproteobacteria (81%) and Alphaproteobacteria contributed 16% of the population (Fig. 15).
Figure 4. CFU/ml of coral mucus calculated from serial dilutions plated on GASW.
Figure 5. Proportion of *Vibrio* spp. was determined based on CFU/ml calculated from GASW and TCBS.
Figure 6. CFU/ml of coral mucus was calculated from serial dilutions plated on GASW media.
Figure 7. Proportion of *Vibrio* spp. was determined based on CFU/ml calculated from GASW and TCBS.
Figure 8. Distribution of bacteria sequenced from healthy *M. capitata* mucus (n= 67). Genera in high abundance are: *Alteromonas* (35%) and *Streptomyces* (28%).
Figure 9. Distribution of bacteria sequenced from acute MWS affected *M. capitata* mucus (n=382). Genera in high abundance are: *Vibrio* (70%) and *Pseudoalteromonas* (10%).
Figure 10. Distribution of bacteria sequenced from chronic MWS affected *M. capitata* mucus (n=418). Genera in high abundance are: *Vibrio* (66%) and *Ruegeria* (10%).
Figure 11. Distribution of *Vibrio* spp. sequenced from MWS affected *M. capitata* mucus (both acute and chronic) (n=544). The species in highest abundance is *Vibrio harveyi* (19%). BLAST searches are only genera specific for 40% of the *Vibrio* spp.
Figure 12. Distribution of bacteria sequenced from the water column (n=28). Genera in high abundance are: *Alteromonas* (13%), *Neptunomonas* (21%), *Pseudoalteromonas* (16%), *Roseobacter* (13%) and *Streptomyces* (16%).
Figure 13. Distribution of taxonomic classes from bacteria sequenced from the water column. The largest proportions are: Actinobacteria (16%), Alphaproteobacteria (13%) and Gammaproteobacteria (59%).
Figure 14. Distribution of taxonomic classes from bacteria sequenced from healthy *M. capitata* mucus. Those in highest abundance are: Actinobacteria (27%), Alphaproteobacteria (12%) and Gammaproteobacteria (59%).
Figure 15. Distribution of taxonomic classes from bacteria sequenced from MWS affected \textit{M. capitata} mucus (both acute and chronic). Those in highest abundance are: Alphaproteobacteria (16\%) and Gammaproteobacteria (81\%).
Growth Curves

Based on the results of the culturable community profiles for healthy and diseased coral, *Alteromonas* sp. (H2 2), *Pseudoalteromonas* sp. (A1 100), *Ruegeria* sp. (C8 3) and *Vibrio harveyi* (C6 2) were selected for challenge experiments. *Pseudoalteromonas* sp., *Ruegeria* sp. and *Vibrio harveyi* were selected as putative pathogens. *V. harveyi* was the most dominant of the *Vibrio* spp. and *Pseudoalteromonas* and *Ruegeria* were second most dominant in acute and chronic MWS, respectively. *Vibrio harveyi* and *Pseudoalteromonas* are Gram-negative Gammaproteobacteria. *Ruegeria* is a Gram-negative Alphaproteobacterium. *Alteromonas* sp. was chosen as a negative bacterial control due to its high proportion on healthy *M. capitata* mucus. It is a Gram-negative rod-shaped Gammaproteobacterium. Growth curves were done in GASW and FSW in either duplicate or triplicate to determine growth kinetics. Knowledge of growth kinetics ensured all bacteria were in the same growth phase at the time of inoculation for challenge experiments. All bacteria were grown up to mid log phase before inoculation above *M. capitata* fragments. *Alteromonas* sp. reached mid log phase at an OD_{600} of approximately 0.090 (Fig. 16). *Pseudoalteromonas* sp. reached mid log phase at an OD_{600} of approximately 0.100 (Fig. 17). *Vibrio harveyi* reached mid log phase at an OD_{600} of approximately 0.200 (Fig. 18). *Ruegeria* sp. reached mid log phase at an OD_{600} of approximately 0.100 (Fig. 19). None of the bacteria selected grew well in FSW (Fig. 16-19).
Figure 16. Growth curve for *Alteromonas* sp. (H2 2) in GASW and FSW. Mid log phase is at approximately 0.090 OD$_{600}$ (indicated by arrow).
Figure 17. Growth curve for *Pseudoalteromonas* sp. (A1 100) in GASW and FSW. Mid log phase is at approximately 0.100 OD$_{600}$ (indicated by arrow).
Figure 18. Growth curve for *Vibrio harveyi* (C2 2) in GASW and FSW. Mid log phase is at approximately 0.200 OD<sub>600</sub> (indicated by arrow).
Figure 19. Growth curve for *Ruiegeria* sp. (C4 3) in GASW and FSW. Mid log phase is at approximately 0.100 OD$_{600}$ (indicated by arrow).
**Bacterial Challenge Experiments**

Five challenge runs were initiated on healthy *M. capitata* fragments in aquaria. The first challenge experiment developed signs of diffuse tissue loss on 80% of the fragments, in addition to all water table fragments within five days. This indicated the corals were unhealthy due to conditions outside the experimental parameters and the challenge was halted. No data from challenge run #1 was included in subsequent data analyses. One important observation from this experiment was the utility of paired water table controls for the duration of the challenge experiment. Mucus was collected from one fragment from each coral colony used in the challenge prior to the start of the experiment and plated to determine starting CFU/ml (Fig. 20).

Challenge runs #2 and #3 used fragments from the same *M. capitata* colonies. Fragments used in challenge run #2 were acclimated in the water table for three days and those used in challenge run #3 were acclimated for two weeks. Three weeks post-inoculation, mucus was collected and plated on GASW and TCBS from all fragments in challenge run #2 to calculate final CFU/ml (Fig. 20). Challenge runs #4 and #5 used fragments from *M. capitata* colonies that were acclimated in the water table for three days pre-inoculation. Fragment acclimation time, mucus pre challenge and concentration of inocula for each challenge run are presented in Table 3.
Figure 20. Average CFU/ml of coral mucus from *M. capitata* fragments pre-inoculation (Pre) and post-inoculation after incubation for 21 days (inoculum identifier on x-axis).
Table 3. CFU/ml from fragments pre-inoculation, identifying fragment number and acclimation time. NG = no growth. (left). CFU/ml of inocula used for each challenge (right).

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<thead>
<tr>
<th>Run #</th>
<th>Fragment Acclimation Time</th>
<th>CFU/ml Pre GASW</th>
<th>CFU/ml Pre TCBS</th>
<th>Inocula</th>
<th>Run #</th>
<th>CFU/ml</th>
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<tbody>
<tr>
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<td>Pseudoalteromonas</td>
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Mucus pre-inoculation had an average of $2.50 \times 10^2$ CFU/ml (Fig. 20). There was minimal variation among fragments in CFU/ml. Only fragments from challenge run #4 had growth on TCBS. These fragments had an average of 6.88% putative *Vibrio* spp. Fragments from challenge runs #2 and #3 were paired to compare three day acclimated fragments versus two week healed fragments. Average CFU/ml of mucus was similar for both sets. Three day fragments had an average of $2.90 \times 10^2$ CFU/ml and two week healed fragments had an average of $2.50 \times 10^2$ CFU/ml.

Mucus collected post-inoculation from control fragments in challenge run #2 had $1.51 \times 10^3$ CFU/ml with no growth on TCBS. Fragments in control tanks were not inoculated. At the end of three weeks, none of the fragments showed signs of tissue loss or tissue thinning (Fig. 21). In challenge run #3, at 23 days post-inoculation, one control fragment exhibited slight tissue thinning.

*Alteromonas* was inoculated onto fragments at mid log phase, which was an average of $6.48 \times 10^8$ CFU/ml. Fragments post challenge with *Alteromonas* from run #2 had an average of $5.00 \times 10^2$ CFU/ml of mucus with no growth on TCBS (Fig. 20). Throughout all four challenge runs, seven fragments showed no signs of tissue thinning or tissue loss for the duration of the three week challenge (Fig. 21). One fragment from run #5 started to bleach at day six but the proportion of discoloration remained steady for the remainder of the challenge. This fragment did not show signs of tissue thinning or tissue loss.
Figure 21. Representative photo of coral fragment pre (A) and post (B) inoculation with no signs of tissue loss. During the course of the challenge, the coral healed over the fragmented edge (indicated by arrow).
\textit{Pseudoalteromonas} was inoculated onto challenge fragments at mid log phase which was an average of $7.86 \times 10^8$ CFU/ml. Fragments post-inoculation with \textit{Pseudoalteromonas} from run #2 had an average of $2.54 \times 10^4$ CFU/ml of mucus with no growth on TCBS (Fig. 20). This is an increase of approximately 100 fold compared to mucus pre-inoculation (Wilcoxon Sign-Rank, n=2, p=0.5). One fragment from each run #3 and #5 challenged with \textit{Pseudoalteromonas} had focal tissue loss that appeared overnight (Fig. 22-A, B, C). The fragment from run #3 had tissue loss occur after three weeks, 23 days post-inoculation (Fig. 22-A). Mucus collected after tissue loss developed had $7.36 \times 10^5$ CFU/ml with $8.2 \times 10^4$ CFU/ml growth on TCBS. Mucus collected from this fragment, after developing tissue loss, had 11\% putative \textit{Vibrio} spp. The fragment from run #5 had tissue loss at 18 days (Fig. 22-B). Mucus collected after tissue loss had $2.15 \times 10^5$ CFU/ml with $2.20 \times 10^4$ CFU/ml growth on TCBS. Mucus collected from this fragment, after developing tissue loss, had 10\% putative \textit{Vibrio} spp. The second fragment from run #5 showed slight diffuse tissue loss at three weeks (Fig. 22-D). Mucus collected from this fragment had $3.42 \times 10^5$ CFU/ml with $6.0 \times 10^4$ CFU/ml growth on TCBS (0\% \textit{Vibrio} spp.).

\textit{Ruegeria} was inoculated onto challenge fragments at mid log phase which was an average of $1.12 \times 10^9$ CFU/ml. Fragments post challenge with \textit{Ruegeria} from run #2 had an average of $5.78 \times 10^3$ CFU/ml of mucus with no growth on TCBS (Fig. 20). This is approximately a 10 fold increase in CFU/ml compared to mucus pre challenge, though not statistically significant (Wilcoxon Sign-Rank, n=2, p=1.0). At three weeks post inoculation with \textit{Ruegeria}, none of the fragments showed signs of tissue thinning or tissue loss (Fig. 21).

\textit{Vibrio harveyi} was inoculated onto challenge fragments at mid log phase which was an average of $2.66 \times 10^9$ CFU/ml. Fragments post challenge with \textit{V. harveyi} from run #2
had an average of $8.9 \times 10^2$ CFU/ml of mucus with no growth on TCBS (Fig. 20). This is a slight increase in CFU/ml as compared to mucus pre challenge but not statistically significant (Wilcoxon Sign-Rank, $n=2$, $p=0.5$).
Figure 22. Coral fragments exhibiting tissue loss while in challenge tanks. (A) Fragment from challenge run #3 developed tissue loss at day 23. (B) Fragment from challenge run #5 developed tissue loss at day 18. (C) Empty skeleton can be seen as a result of the tissue loss which appears to be peeling away from the skeleton. (D) The second fragment from run #5 showed diffuse tissue loss after 20 days. Tissue is lighter in color and skeletal elements are poking out from the edges.
DISCUSSION

This study provides the first characterization of culturable bacteria profiles for *M. capitata* and MWS. Identifying the normal flora of healthy *M. capitata* provides a baseline for etiology studies. Shifts in community structure may be identified by comparing bacterial population structure in diseased mucus compared to that of healthy mucus. These profiles also provide a baseline for future studies looking into the disease processes of MWS. Identification of antimicrobials produced by healthy-associated bacteria may provide insight into a possible protective role they may serve in healthy corals (Koren & Rosenberg 2006). Identification of virulence factors produced by bacteria cultured from disease mucus may provide insight into the processes involved that allow these bacteria to colonize mucus from MWS affected corals.

Healthy and diseased mucus-associated bacterial profiles allowed for thoughtful selection of bacteria for use in challenge experiments. Challenge experiments for coral disease need to be optimized for the coral species and disease under investigation, especially when challenge conditions include artificial environments. Fragments have been removed from their natural environment and placed in closed-system aquaria filled with FSW. The stress of challenge parameters may allow some bacteria to become opportunistic pathogens. *Alteromonas* was selected as a negative bacterial control because of its elevated numbers in healthy mucus. The use of a negative bacterial control provides a challenge where no disease signs should be observed. Water table controls allows fragments to be monitored for external water factors that may skew challenge results. Coral colonies may be already stressed before collection and, therefore, pre-disposed to disease. The water table may also expose the fragments to stressors or potentially detrimental bacteria.
Knowing the growth kinetics for bacterial strains being used in challenge experiments provides consistency in inocula from one challenge to the next. Growth curves also ensure all bacteria are in the same growth phase before inoculation in aquaria. Previous etiology studies have inoculated using bacteria grown to a specified OD or for an approximate number of hours (Banin et al. 2003; Ben-Haim et al. 2003). These methods may result in inocula being at various growth phases and cell densities. Rinsing bacterial cultures in FSW prior to inoculation removed media from the culture. This process minimized excess nutrient loading which has been associated with coral disease (Kaczmarsky et al. 2005).

Current methods being employed for challenge experiments involve varying fragment acclimation times (Patterson et al. 2002; Banin et al. 2003). There was no statistical difference between CFU/ml from paired three day versus two week acclimated fragments (Wilcoxon Sign-Rank, n=2, p=1.0) for *M. capitata* experiments. Challenge fragments may have similar CFU/ml but it is unknown whether the bacterial community structure shifts during acclimation time in the water table. Mucus sloughing is a stress response in corals (Rublee et al. 1980). After sloughing their mucus, bacterial colonization of the new layer produced while in the water table may be different than prior to fragmentation.

Challenge experiments indicate *Pseudoalteromonas* may or may not be associated with tissue loss in *M. capitata* fragments. Tissue loss occurred in two challenge runs. Tissue loss from challenge run #3 occurred just over three weeks, 23 days. Because three weeks had expired, at the time of tissue loss five days had passed since the previous water change. Tank effect may have factored into the occurrence of tissue loss since at 23 days
one of the control fragments showed signs of tissue thinning. Mucus collected from fragments inoculated with *Pseudoalteromonas* had a 100 fold increase in CFU/ml. This may indicate colonization of the mucus by *Pseudoalteromonas* although this cannot be verified without bacterial identification. Challenge runs do not indicate definitively that *Pseudoalteromonas* induces focal tissue loss in *M. capitata*. Further investigation into the role *Pseudoalteromonas* may play in the pathogenesis of MWS is required.

A culture-dependant approach to coral disease does not identify all bacteria present in the mucus layer but it provides a starting place for identifying normal mucosal flora. From this baseline population, shifts in community structure associated with diseased corals may be identified. Recognizing these shifts provides a guideline for selection of culturable putative pathogens for use in challenge experiments. Identifying shifts in community structure may provide insight into the disease process in corals. For example, shifts toward sediment-associated bacteria may suggest sediment stress as the initiator of the disease. Shifts toward fecal enteric bacteria may indicate sewage runoff as the initiator of the disease (Patterson *et al.* 2002).

Despite the issues with using culture-dependant methods, the data from this study is consistent with previous reports suggesting a specific association with the normal bacterial flora and healthy mucus (Koren & Rosenberg 2006). The water and healthy mucus community have similar culturable CFU/ml, but different population structures. The culturable population of the surrounding water column was diverse and not dominated by any one genera. In contrast, healthy mucus was colonized by a high proportion of *Alteromonas* and *Streptomyces*. Both of these genera are known to produce a wide range of antimicrobial compounds which may inhibit colonization of the mucus by other potentially
detrimental bacteria. *Alteromonas* has been shown to produce compounds that inhibit growth of *Pseudoalteromonas* and *Vibrio* (Long et al. 2003). *Streptomyces* has been shown to inhibit biofilm formation by *Vibrio* spp. and inhibit quorum sensing by *V. harveyi* (Long et al. 2003; You et al. 2007).

Also in agreement with previously published observations, mucus from diseased corals had an increase in CFU/ml as well as a shift to a *Vibrio* dominated community (Thompson et al. 2004; Ritchie 2006; Dinsdale et al. 2008; Rosenberg & Ben-Haim 2002; Breitbart et al. 2005). One example of this is the increase in culturable *Vibrio* spp. associated with skeletal tumors on *Porites compressa* (Breitbart et al. 2005). MWS affected mucus had approximately a 100 fold increase in CFU/ml and a 17.7% increase in *Vibrio* spp. compared to healthy mucus. The most predominant of the *Vibrio* spp. represented in MWS mucus was *V. harveyi*. *Vibrio harveyi* is pathogenic in shrimp, abalone and many fish species (Austin & Zhang, 2006; Alavandi et al. 2006; Sawabe et al. 2007). While *V. harveyi* has been discussed as a potential coral pathogen (Weil et al. 2006), to date this bacterium has not been positively identified as an etiologic agent of coral disease. The observation that no colonies grew on TCBS post-challenge may indicate that the increase in culturable *Vibrio* spp. seen in MWS in the field is opportunistic colonization.

During this study, approximately 15% of the colonies selected from diseased GASW plates did not grow out when transferred to fresh GASW plates or broth. During the period of time when stocks of bacteria were being made, the temperature sensor in the shaker lost normal function, elevating the temperature to approximately 38°C. A low proportion of cultures were viable after this temperature shock. This non-archived set of samples was therefore not available for sequencing for community profiles. If these
isolates had been identified, the population structure of MWS might have a slightly different composition than what was identified. This non-identified proportion may have contributed to the difference in proportion of \textit{Vibrio} spp. determined by plate counts and sequence analysis, 30.1\% and 68.0\%, respectively (not statistically significant, Wilcoxon Sign-Rank, \(n=2\), \(p=0.5\)).

\textit{Pseudoalteromonas} and \textit{Ruegeria} were also found in elevated proportions in MWS mucus. Species of \textit{Pseudoalteromonas} are known to be pathogenic to fish (Jesus Pujalte \textit{et al.} 2007). \textit{Pseudoalteromonas tunicata} has been shown to remove preestablished biofilms by producing antimicrobials (Rao \textit{et al.} 2005). Production of these or similar antimicrobial compounds may allow \textit{Pseudoalteromonas} to colonize MWS affected mucus. To date, neither \textit{Pseudoalteromonas} nor \textit{Ruegeria} have been identified as etiologic agents of coral disease.

While a number of important observations were made during this study, there are no clear conclusions relating to the etiology of MWS. Helminth infestation, healing, metazoan and ciliates are all factors that have been associated with MWS by histology (Aeby HCRI-RP final report 2007). Thus, histology results and bacterial analysis illustrates the wide dynamic of factors involved with MWS. Three fragments from each acute and chronic tagged colonies from this study were collected for histological analysis (data not shown). Helminth infestation, the presence of unidentified metazoans and signs of healing were all identified in the histology results. As a result, it seems unlikely that specific histologic features will be associated with acute or chronic MWS.

Challenge experiments used to determine etiologies also identify parameters and unknowns that make Koch's postulates difficult to fulfill for coral diseases (Ritchie \textit{et al.}}
The postulates do not take into consideration etiologic consortiums, non-culturable etiologic agents, changes in host susceptibility, or increases in bacterial virulence associated with environmental changes (Lesser et al. 2007). In addition, challenge experiments with corals require that fragments are removed from the colony and natural environment and placed in closed aquaria systems (Ritchie et al. 2001). This artificial environment stress may increase the coral's susceptibility to disease. Finally, as seen in challenge run #1, unknown factors external to challenge parameters may contribute to the decline of coral health in challenge experiments. Optimization of challenge methods for specific coral diseases may help combat some of these complications in fulfilling Koch's postulates.

The community profiles generated through this research for healthy and MWS affected M. capitata provides direction for future studies. Additional challenge runs need to be completed to identify whether or not Pseudoalteromonas induces tissue loss similar to MWS. If Pseudoalteromonas is pathogenic to M. capitata, controlling for specific stressors in challenge runs may indicate conditions necessary for initiation of the disease (Lesser et al. 2007).

Identification of antimicrobial compounds or virulence factors produced by key bacterial profile elements will provide insight into bacteria-bacteria interactions within the mucus layer. Factors produced by healthy-associated bacteria may provide a link to their role in coral's resistance to disease. Factors produced by MWS-associated bacteria may also provide insight into the disease process and how these shifts are molded. As in Vibrio induced bleaching, temperature induced up-regulation of virulence factors may provide insight into disease initiation (Ben-Haim et al. 2003; Banin et al. 2001). More in depth
investigation into the defenses *M. capitata* has against pathogens will increase our knowledge of the MWS disease process.

Using culture-independent techniques to further characterize bacterial communities for healthy *M. capitata* and MWS affected mucus will combat some of the previously stated concerns relating to the use of culture-dependant methods. Suitable methods that may be applied to this question may include the use of clone libraries, DNA fingerprinting methods (i.e. Terminal Restriction Fragment Length Polymorphism) or microscopy methods (i.e. Fluorescence *in situ* Hybridization) (Osborn *et al.* 2000; Dorigo *et al.* 2005). Investigations into coral disease using culture-independent techniques have indicated some divergent conclusions from those described using culture-dependant techniques (Cooney *et al.* 2002; Sekar *et al.* 2006). While these methods yield a more complete profile, they also have some bias. For example, clone libraries have been described as having utility for identifying uncultured bacteria from coral samples (Cooney *et al.* 2002) but in reality this method may also be biased since cloning vectors may preferentially incorporate individual bacterial inserts (Acinas *et al.* 2005). No single method will provide an all-inclusive profile but combining methods will present a more complete picture.

In conclusion, this study identifies for the first time the culturable bacterial community profiles for healthy *M. capitata* and MWS affected *M. capitata*. It is the first study to attempt to initiate MWS *in vitro* using bacteria found in elevated numbers in MWS affected mucus samples. Finally, preliminary challenge experiments suggest that while Koch's postulates were not fulfilled during this study, there is evidence that a culturable bacterium may be associated with initiation of MWS.
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Appendix 1 - GASW Recipe and PCR Specifics

Glycerol Artificial Seawater Media
*Add 1L of DI H₂O to ingredients and autoclave

20.8g NaCl – Sodium Chloride
0.56g KCl – Potassium Chloride
4.8g MgSO₄ 7H₂O – Magnesium Sulfate Heptahydrate
4.0g MgCl₂ 6H₂O – Magnesium Chloride Hexahydrate
0.01g K₂HPO₄ – Potassium Phosphate Dibasic
0.001g FeSO₄ 7H₂O – Iron (II) Sulfate Heptahydrate
2.0g Instant Ocean (Aquarium Systems, Inc. Mentor OH)
0.48g Tris Base
4.0g Peptone
2.0g Yeast Extract
2.0ml Glycerol
15-20g Agar (omit for liquid media)

16S rDNA primers

8F- 5’-AGAGTTTGATCATGGCTCAG-3’

1513R- 5’-TACGGTTACCTTGTTACGACTT-3’

(Sorokin et al. 2001)

PCR conditions
Cycle 1: 95° 4 minutes
Cycle 2: 95° 30 seconds (35x)
            55° 30 seconds
            72° 90 seconds
Cycle 3: 72° 7 minutes
         12° Hold

(Sorokin et al. 2001)
## Appendix 2 – Bacterial Sequence Results

### Water Isolates

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| C2 24 | **Alteromonas** sp. | Gammaproteobacteria | EF491997 |
| C2 24 | **Vibrio** *harveyi* | Gammaproteobacteria | EU090704 |
| C2 25 | **Silicibacter** sp. | Alphaproteobacteria | EU267638 |
| C2 26 | **Pseudoalteromonas** sp. | Gammaproteobacteria | EF587966 |
| C2 26 | **Silicibacter** sp. | Alphaproteobacteria | EU267638 |
| C2 27 | **Vibrio** *fortis* | Gammaproteobacteria | AJ514917 |
| C2 28 | **Alteromonas alvinellae** | Gammaproteobacteria | AF288360 |
| C2 28 | **Staphylococcus hominis** | Bacillales | EF512729 |
| C2 29 | **Pseudoalteromonas** sp. | Gammaproteobacteria | EU167384 |
| C2 29 | **Vibrio** *harveyi* | Gammaproteobacteria | AM422802 |
| C2 3 | **Alteromonas** sp. | Gammaproteobacteria | DQ107395 |
| C2 3 | **Thalassomonas** sp. | Gammaproteobacteria | EF657834 |
| C2 30 | **Vibrio** *aestuarianus* | Gammaproteobacteria | DQ985231 |
| C2 30 | **Vibrio** *cyclitrophlicus* | Gammaproteobacteria | EF094886 |
| C2 31 | **Vibrio** *harveyi* | Gammaproteobacteria | DQ995248 |
| C2 32 | **Vibrio** *algilolyticus* | Gammaproteobacteria | AM921804 |
| C2 32 | **Vibrio** sp. | Gammaproteobacteria | EF587973 |
| C2 33 | **Vibrio** sp. | Gammaproteobacteria | EF587973 |
| C2 34 | **Vibrio** sp. | Gammaproteobacteria | EF587973 |
| C2 35 | **Vibrio** sp. | Gammaproteobacteria | EF587973 |
| C2 36 | **Vibrio** sp. | Gammaproteobacteria | EF587973 |
| C2 37 | **Vibrio** sp. | Gammaproteobacteria | EF587973 |
| C2 38 | **Vibrio** *sinoensis* | Gammaproteobacteria | DQ451210 |
| C2 39 | **Ruegeria** sp. | Alphaproteobacteria | EF619092 |
| C2 4 | **Alteromonas** sp. | Gammaproteobacteria | DQ107397 |
| C2 40 | **Ruegeria** sp. | Alphaproteobacteria | AM709695 |
| C2 40 | **Vibrio** *lentus* | Gammaproteobacteria | AY254042 |
| C2 41 | **Vibrio** sp. | Gammaproteobacteria | EF587973 |
| C2 42 | **Vibrio** *cyclitrophlicus* | Gammaproteobacteria | AM422804 |
| C2 43 | **Vibrio** sp. | Gammaproteobacteria | EF587973 |
| C2 44 | **Vibrio** sp. | Gammaproteobacteria | EF587973 |
| C2 45 | **Vibrio** *cyclitrophlicus* | Gammaproteobacteria | AM422804 |
| C2 46 | **Vibrio** *aestuarianus* | Gammaproteobacteria | DQ985231 |
| C2 47 | **Vibrio** *aestuarianus* | Gammaproteobacteria | DQ985231 |
| C2 47 | **Vibrio** *fortis* | Gammaproteobacteria | AJ514917 |
| C2 48 | **Alteromonas macleodii** | Gammaproteobacteria | AM887685 |
| C2 48 | **Vibrio** sp. | Gammaproteobacteria | EF587973 |
| C2 49 | **Vibrio** sp. | Gammaproteobacteria | DQ903973 |
| C2 49 | **Vibrio** sp. | Gammaproteobacteria | EF587973 |
| C2 5 | **Ruegeria** sp. | Alphaproteobacteria | AM709695 |
| C2 5 | **Vibrio** *lentus* | Gammaproteobacteria | EF178477 |
| C2 50 | **Vibrio** sp. | Gammaproteobacteria | EF587973 |
| C2 51 | **Vibrio** *cyclitrophlicus* | Gammaproteobacteria | AM422804 |
| C2 52 | **Alteromonas genoviensis** | Gammaproteobacteria | AM887686 |
| C2 52 | **Vibrio** *corallilyticus* | Gammaproteobacteria | EF114163 |
| C2 53 | **Ruegeria** sp. | Alphaproteobacteria | AM709695 |
| C2 54 | **Vibrio** sp. | Gammaproteobacteria | EF587973 |
| C2 55 | Ruegeria sp. | Alphaproteobacteria | AM709695 |
| C2 56 | Vibrio sp. | Gammaproteobacteria | EF033382 |
| C2 57 | Vibrio sp. | Gammaproteobacteria | EF587973 |
| C2 58 | Vibrio sp. | Gammaproteobacteria | EF587973 |
| C2 59 | Vibrio alginolyticus | Gammaproteobacteria | AM921804 |
| C2 59 | Vibrio cyclitrophicus | Gammaproteobacteria | DQ481610 |
| C2 6 | Vibrio cyclitrophicus | Gammaproteobacteria | AM422804 |
| C2 6 | Vibrio fortis | Gammaproteobacteria | DQ437752 |
| C2 60 | Staphylococcus sp. | Bacilli | EU169558 |
| C2 60 | Vibrio cyclitrophicus | Gammaproteobacteria | AM422804 |
| C2 61 | Staphylococcus sp. | Bacilli | AM778692 |
| C2 61 | Vibrio sp. | Gammaproteobacteria | EF584020 |
| C2 63 | Alteromonas macleodii | Gammaproteobacteria | AM887685 |
| C2 63 | Vibrio sp. | Gammaproteobacteria | EF587973 |
| C2 64 | Silicibacter sp. | Alphaproteobacteria | EF657830 |
| C2 65 | Vibrio sp. | Gammaproteobacteria | EF587973 |
| C2 66 | Vibrio cyclitrophicus | Gammaproteobacteria | AM422804 |
| C2 67 | Vibrio cyclitrophicus | Gammaproteobacteria | AM709695 |
| C2 68 | Vibrio sp. | Gammaproteobacteria | EF587973 |
| C2 69 | Vibrio sp. | Gammaproteobacteria | EF587973 |
| C2 7 | Alteromonas sp. | Gammaproteobacteria | DQ107395 |
| C2 7 | Vibrio fortis | Gammaproteobacteria | AJ514917 |
| C2 70 | Vibrio cyclitrophicus | Gammaproteobacteria | AM422804 |
| C2 71 | Vibrio cyclitrophicus | Gammaproteobacteria | AM709695 |
| C2 72 | Vibrio sp. | Gammaproteobacteria | DQ985231 |
| C2 73 | Vibrio sinaloensis | Gammaproteobacteria | DQ451211 |
| C2 74 | Vibrio lentus | Gammaproteobacteria | EF178477 |
| C2 75 | Vibrio harveyi | Gammaproteobacteria | EU090704 |
| C2 75 | Vibrio sp. | Gammaproteobacteria | DQ985231 |
| C2 76 | Vibrio sp. | Gammaproteobacteria | EF587973 |
| C2 77 | Vibrio sp. | Gammaproteobacteria | DQ985231 |
| C2 78 | Vibrio sp. | Gammaproteobacteria | EF587973 |
| C2 79 | Vibrio sp. | Gammaproteobacteria | EF587973 |
| C2 8 | Vibrio sinaloensis | Gammaproteobacteria | DQ451210 |
| C2 80 | Alteromonas macleodii | Gammaproteobacteria | AM887685 |
| C2 81 | Silicibacter sp. | Alphaproteobacteria | EF033456 |
| C2 82 | Vibrio sp. | Gammaproteobacteria | EU253580 |
| C2 83 | Vibrio aestuarianus | Gammaproteobacteria | DQ985231 |
| C2 83 | Vibrio harveyi | Gammaproteobacteria | EU090704 |
| C2 84 | Vibrio alginolyticus | Gammaproteobacteria | AM921804 |
| C2 84 | Vibrio harveyi | Gammaproteobacteria | EU196615 |
| C2 85 | Vibrio aestuarianus | Gammaproteobacteria | DQ985231 |
| C2 85 | Vibrio sp. | Gammaproteobacteria | EF587973 |
| C2 86 | Vibrio aestuarianus | Gammaproteobacteria | DQ985231 |
| C2 87 | Vibrio aestuarianus | Gammaproteobacteria | DQ985231 |
| C2 87 | Vibrio aestuarianus | Gammaproteobacteria | DQ985231 |
| C2 88 | Vibrio sp. | Gammaproteobacteria | DQ985231 |
| C2 89 | Vibrio fortis | Gammaproteobacteria | EF584063 |
| C2 89 | Vibrio sp. | Gammaproteobacteria | DQ437752 |
| C2 89 | Vibrio sp. | Gammaproteobacteria | EF587973 |
C2  9  Alteromonas sp.  Gammaproteobacteria  AJ784107
C2  90 Ruegeria sp.  Alphaproteobacteria  AM709735
C2  91 Vibrio sp.  Gammaproteobacteria  EF587973
C2  92 Vibrio sp.  Gammaproteobacteria  EU253580
C2  93 Vibrio sp.  Gammaproteobacteria  EU259885
C2  94 Thalassomonas sp.  Gammaproteobacteria  EF588011
C2  95 Vibrio sp.  Gammaproteobacteria  EF587973
C2  96 Vibrio corallilyticus  Gammaproteobacteria  EF114163
C2  97 Vibrio corallilyticus  Gammaproteobacteria  EF114163
C2  98 Vibrio corallilyticus  Gammaproteobacteria  EF094886
C2  99 Vibrio corallilyticus  Gammaproteobacteria  AM422804
C217 Alteromonas macleodii  Gammaproteobacteria  AM887685
C218 Alteromonas sp.  Gammaproteobacteria  DQ107397
C219 Alteromonas sp.  Gammaproteobacteria  DQ107397
C3   1 Pseudoalteromonas sp.  Gammaproteobacteria  EU167384
C3   10 Vibrio corallilyticus  Gammaproteobacteria  AM921804
C3   11 Acinetobacter sp.  Gammaproteobacteria  EU276980
C3   12 Pseudoalteromonas sp.  Gammaproteobacteria  AB243250
C3   13 Vibrio cyclitrophicus  Gammaproteobacteria  AJ440004
C3   14 Vibrio harveyi  Gammaproteobacteria  DQ437752
C3   16 Vibrio aestuarianus  Gammaproteobacteria  EF588011
C3   17 Vibrio fortis  Gammaproteobacteria  AJ514916
C3   18 Shewanella sp.  Gammaproteobacteria  EF587949
C3   19 Vibrio harveyi  Gammaproteobacteria  AJ514917
C3   2 Vibrio harveyi  Gammaproteobacteria  AJ514916
C3   3 Psychrobacter sp.  Gammaproteobacteria  EU143362
C3   4 Pseudoalteromonas sp.  Gammaproteobacteria  EU167384
C3   5 Ruegeria sp.  Alphaproteobacteria  AM709695
C3   9 Vibrio harveyi  Gammaproteobacteria  AM709695
C4   1 Acinetobacter sp.  Gammaproteobacteria  EU276980
C4  100 Staphylococcus sp.  Bacilli  AM903333
C4  101 Silicibacter sp.  Alphaproteobacteria  EF588011
C4  102 Vibrio proteolyticus  Gammaproteobacteria  DQ005240
C4  103 Vibrio sp.  Gammaproteobacteria  EF587973
C4  104 Ruegeria sp.  Alphaproteobacteria  AM709695
C4  105 Ruegeria sp.  Alphaproteobacteria  AM422804
C4  106 Ruegeria sp.  Alphaproteobacteria  AM709695
C4  108 Ruegeria sp.  Alphaproteobacteria  AM709695
C4  109 Bacillus sp.  Bacilli  DQ923215
C4  111 Ruegeria sp.  Alphaproteobacteria  AM709695
C4  113 Staphylococcus sp.  Bacilli  DQ448767
C4  115 Vibrio sp.  Gammaproteobacteria  EU253580
C4  15 Ruegeria sp.  Alphaproteobacteria  AM709695
C4  16 Roseobacter sp.  Alphaproteobacteria  AY371428
C4  18 Vibrio sinaloensis  Gammaproteobacteria  EU043382
C4   2 Ruegeria sp.  Alphaproteobacteria  AM709699
C4  20 Vibrio sinaloensis  Gammaproteobacteria  EU043382
C4  22 Ruegeria sp.  Alphaproteobacteria  AM709695
C4  23 Ruegeria sp.  Alphaproteobacteria  AM709695

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| C4 24 | *Ruegeria* sp. | Alphaproteobacteria | DQ917855 |
| C4 25 | *Vibrio sinaloensis* | Gammaproteobacteria | EU043382 |
| C4 26 | *Vibrio vulnificus* | Gammaproteobacteria | AY264936 |
| C4 27 | *Vibrio* sp. | Gammaproteobacteria | EF587981 |
| C4 28 | *Vibrio sinaloensis* | Gammaproteobacteria | DQ451211 |
| C4 29 | *Vibrio sinaloensis* | Gammaproteobacteria | EU043382 |
| C4 30 | *Ruegeria* sp. | Alphaproteobacteria | AY494623 |
| C4 31 | *Pseudomonas* sp. | Gammaproteobacteria | EU440046 |
| C4 32 | *Roseobacter* sp. | Alphaproteobacteria | EU440046 |
| C4 33 | *Vibrio* sp. | Gammaproteobacteria | AM422804 |
| C4 34 | *Vibrio cyclitrophicus* | Gammaproteobacteria | AM422804 |
| C4 35 | *Vibrio proteolyticus* | Gammaproteobacteria | DQ995521 |
| C4 36 | *Vibrio* sp. | Gammaproteobacteria | EU259885 |
| C4 37 | *Vibrio proteolyticus* | Gammaproteobacteria | DQ995521 |
| C4 38 | *Ruegeria* sp. | Alphaproteobacteria | DQ917855 |
| C4 39 | *Vibrio* sp. | Gammaproteobacteria | EF587973 |
| C4 40 | *Vibrio* sp. | Gammaproteobacteria | EF587973 |
| C4 41 | *Vibrio* sp. | Gammaproteobacteria | EF587973 |
| C4 42 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 43 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 44 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 45 | *Ruegeria* sp. | Alphaproteobacteria | DQ917819 |
| C4 46 | *Vibrio* sp. | Gammaproteobacteria | EF587973 |
| C4 47 | *Vibrio* sp. | Gammaproteobacteria | EF587973 |
| C4 48 | *Vibrio* sp. | Gammaproteobacteria | EF587973 |
| C4 49 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 50 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 51 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 52 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 53 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 54 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 55 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 56 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 57 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 58 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 59 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 60 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 61 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 62 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 63 | *Vibrio* sp. | Gammaproteobacteria | DQ521084 |
| C4 64 | *Ruegeria* sp. | Alphaproteobacteria | AY136122 |
| C4 65 | *Roseobacter* sp. | Alphaproteobacteria | AY136122 |
| C4 66 | *Vibrio* sp. | Gammaproteobacteria | AY136122 |
| C4 67 | *Vibrio* sp. | Gammaproteobacteria | AY136122 |
| C4 68 | *Vibrio* sp. | Gammaproteobacteria | AY136122 |
| C4 69 | *Ruegeria* sp. | Alphaproteobacteria | AY136122 |
| C4 70 | *Ruegeria* sp. | Alphaproteobacteria | AM709695 |
| C4 70 | *Ruegeria* sp. | Alphaproteobacteria | AM709695 |
| C4 71 | *Ruegeria* sp. | Alphaproteobacteria | DQ917819 |
| C4 72 | *Thalassobius* sp. | Gammaproteobacteria | EU090254 |
| C4 73 | *Vibrio sinaloensis* | Gammaproteobacteria | EU043381 |
| C4 74 | *Vibrio* sp. | Gammaproteobacteria | AM709695 |
| C4 75 | *Ruegeria* sp. | Alphaproteobacteria | DQ917819 |
| C4 76 | *Vibrio sinaloensis* | Gammaproteobacteria | EU043381 |
| C4 77 | *Vibrio* sp. | Gammaproteobacteria | EU267645 |
| C4 78 | *Vibrio* sp. | Gammaproteobacteria | AM709695 |
| C4 79 | Roseobacter sp. | Alphaproteobacteria | EU090129 |
| C4 80 | Roseobacter sp. | Alphaproteobacteria | EU090129 |
| C4 81 | Roseobacter sp. | Alphaproteobacteria | EU090129 |
| C4 82 | *Staphylococcus cohnii* | Bacilli | EU090129 |
| C4 83 | Rhodobacteraceae | Alphaproteobacteria | DQ403810 |
| C4 84 | *Vibrio* sp. | Gammaproteobacteria | EU267645 |
| C4 85 | *Vibrio* sp. | Gammaproteobacteria | AM709695 |
| C4 86 | *Vibrio* sp. | Gammaproteobacteria | AM709695 |
| C4 87 | *Vibrio* sp. | Gammaproteobacteria | AM709695 |
| C4 88 | *Vibrio* sp. | Gammaproteobacteria | AM709695 |
| C4 89 | *Ruegeria* sp. | Alphaproteobacteria | EF619092 |
| C4 90 | *Ruegeria* sp. | Alphaproteobacteria | EF619092 |
| C4 91 | *Ruegeria* sp. | Alphaproteobacteria | EF619092 |
| C4 92 | Alphaproteobacteria | Alphaproteobacteria | AM709695 |
| C4 93 | *Thalassobius* sp. | Alphaproteobacteria | EF587959 |
| C4 94 | *Ruegeria* sp. | Alphaproteobacteria | EF619092 |
| C4 95 | *Ruegeria* sp. | Alphaproteobacteria | EF619092 |
| C4 96 | *Ruegeria* sp. | Alphaproteobacteria | EF619092 |
| C4 97 | Alphaproteobacteria | Alphaproteobacteria | AM709695 |
| C4 98 | Alphaproteobacteria | Alphaproteobacteria | AM709695 |
| C4 99 | Alphaproteobacteria | Alphaproteobacteria | AM709695 |
| C5 10 | Roseobacter sp. | Alphaproteobacteria | AM709695 |
| C5 11 | Staphylococcus sp. | Bacilli | EU090254 |
| C5 12 | Roseobacter sp. | Alphaproteobacteria | AM709695 |
| C5 13 | *Vibrio corallilyticus* | Gammaproteobacteria | DQ079633 |
| C5 14 | *Vibrio corallilyticus* | Gammaproteobacteria | EF094886 |
| C5 15 | *Vibrio sinaloensis* | Gammaproteobacteria | EU043382 |
| C5 16 | *Vibrio corallilyticus* | Gammaproteobacteria | EF094886 |
| C5 17 | *Vibrio* sp. | Gammaproteobacteria | AM709695 |
| C5 18 | *Vibrio* sp. | Gammaproteobacteria | AM709695 |
| C5 19 | *Vibrio* sp. | Gammaproteobacteria | AM709695 |
| C5 20 | Pseudoalteromonas sp. | Gammaproteobacteria | EU167384 |
| C5 21 | Roseobacter sp. | Alphaproteobacteria | AM709695 |
| C5 22 | *Vibrio harveyi* | Gammaproteobacteria | DQ079633 |
| C5 23 | Roseobacter sp. | Alphaproteobacteria | AM709695 |
| C5 24 | Roseobacter sp. | Alphaproteobacteria | AM709695 |
| C5 25 | *Vibrio* sp. | Gammaproteobacteria | AM709695 |
| C5 26 | Pseudoalteromonas sp. | Gammaproteobacteria | AM709695 |
| C5 27 | Roseobacter sp. | Alphaproteobacteria | EU167384 |
| C5 28 | *Vibrio harveyi* | Gammaproteobacteria | AM422802 |
| C5 29 | Roseobacter sp. | Alphaproteobacteria | EF414227 |
| C5 30 | Roseobacter sp. | Alphaproteobacteria | AM709695 |
| C5 31 | *Vibrio* sp. | Gammaproteobacteria | AM709695 |
| C5 32 | Roseobacter sp. | Alphaproteobacteria | AM709695 |
| C5 33 | Pseudoalteromonas sp. | Gammaproteobacteria | EF491997 |
| C5 34 | *Vibrio* sp. | Gammaproteobacteria | EU440046 |
| C5 35 | *Vibrio* sp. | Gammaproteobacteria | DQ521081 |
| C5 36 | Roseobacter sp. | Alphaproteobacteria | AM709695 |
| C5 37 | Vibrio sp. | Gammaproteobacteria | EU440046 |
| C5 4  | Ruegeria sp. | Alphaproteobacteria | AM709695 |
| C5 40 | Vibrio sp. | Gammaproteobacteria | EU440046 |
| C5 41 | Vibrio sp. | Gammaproteobacteria | AY136110 |
| C5 43 | Vibrio sp. | Gammaproteobacteria | EU440046 |
| C5 44 | Vibrio sp. | Gammaproteobacteria | AY136110 |
| C5 45 | Vibrio vulnificus | Gammaproteobacteria | AY264936 |
| C5 46 | Vibrio sp. | Gammaproteobacteria | DQ521081 |
| C5 47 | Vibrio sp. | Gammaproteobacteria | EU440046 |
| C5 48 | Vibrio sp. | Gammaproteobacteria | DQ521081 |
| C5 49 | Vibrio sp. | Gammaproteobacteria | AY136110 |
| C5 50 | Vibrio sp. | Gammaproteobacteria | DQ521081 |
| C5 51 | Roseobacter sp. | Alphaproteobacteria | AY663966 |
| C5 53 | Vibrio sp. | Gammaproteobacteria | AY136110 |
| C5 54 | Vibrio sp. | Gammaproteobacteria | EU440046 |
| C5 55 | Vibrio sp. | Gammaproteobacteria | AY136110 |
| C5 56 | Vibrio sp. | Gammaproteobacteria | AY136110 |
| C5 57 | Roseobacter sp. | Alphaproteobacteria | AY663966 |
| C5 58 | Vibrio sp. | Gammaproteobacteria | AY374396 |
| C5 59 | Roseobacter sp. | Alphaproteobacteria | EF414227 |
| C5 60 | Curtobacterium sp. | Actinobacteridae | EU236753 |
| C5 61 | Roseobacter sp. | Alphaproteobacteria | EF414227 |
| C5 62 | Vibrio sp. | Gammaproteobacteria | EU440046 |
| C5 63 | Rhodobacteraceae | Alphaproteobacteria | DQ403810 |
| C5 64 | Vibrio sp. | Gammaproteobacteria | DQ521081 |
| C5 8  | Roseobacter sp. | Alphaproteobacteria | EF414227 |