UNIVERSITY OF CALIFORNIA, SAN DIEGO

Microbial Responses and Coral Reef Resilience to Organic Matter Inputs

A dissertation submitted in partial satisfaction of the

requirements for the degree of Doctor of Philosophy

in

Marine Biology

by

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The dissertation of Melissa Sara Garren is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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DEDICATION

This dissertation is dedicated to the many microbes and coral polyps who sacrificed their lives for the sake of my knowledge, and to my husband, family and friends who I can never thank enough for being their amazing, wonderful selves.

EPIGRAPH

As we like to say around here...

Seeing is believing.

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PUBLICATIONS

M. Garren and F. Azam (2010) A New Method for Counting Bacteria Associated with Coral Mucus. Applied and Environmental Microbiology 76: 6128-6133

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ABSTRACT OF THE DISSERTATION

Microbial Responses and Coral Reef Resilience to Organic Matter Inputs

by

Melissa Sara Garren

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Professor Farooq Azam, Chair

Little attention has been given to the small-scale mechanisms relevant to microbial processes that determine the resilience of individual corals to the stress of organic matter (OM) inputs. Such mechanisms may be a critical link for predicting larger scale patterns of reef resilience. The research presented here aims to develop methods necessary to elucidate these processes and to explore the *in situ* responses of microbial assemblages on coral reefs experiencing persistent OM enrichment.

A new method using trypsinization of coral mucus before staining for epifluorescence microscopy is described. It is then applied to a coral reef ecosystem influenced by sewage effluent to discover that corals exposed to effluent had the same number of bacteria present as reference corals; however, the size structure of the community was significantly different.

Investigating the responses of microbial communities (from both the water column and corals) to OM inputs from coastal milkfish (*Chanos chanos*) pens, we found that the percentage of the water bacterial community attached to particles increased by more than 50-fold near the pens. This suggested a physiological or life-strategy change may be induced by such enrichment. A clonally replicated coral transplantation experiment examined the response of naïve coral-associated bacterial communities to high and low levels of pen effluent exposure. We found that the communities on corals exposed to high levels of effluent had drastically altered community compositions after five days and the abundance of bacteria in the coral mucus-tissue slurries were ~100-fold higher controls at low effluent and reference sites. We also observed a surprising resilience of these communities in that their composition and total abundance recovered by day 22.

A combination of novel imaging technology (high speed laser scanning confocal microscopy on live coral) and controlled aquaria experiments were developed and used to investigate a microscale mechanism by which such resilience may occur: that corals may release ("shed") bacteria into the surrounding water as a mechanism for controlling bacterial abundance on their surface. We observed this phenomenon in real time, and quantified an increase in the rate at which corals shed bacteria as a response to OM enrichment.

Chapter 1

Introduction: Setting the Stage

Our understanding of the roles that microbes play in the coral holobiont (the animal and its associated microbiota; Rohwer et al 2002) has dramatically expanded in the past 10 years, in large part due the accessibility of molecular methods (Ainsworth et al 2010, Knowlton and Rohwer 2003). There have also been persistent efforts to image the holobiont, though these have been slower and more difficult. Corals present a suite of challenges for imaging that include a sticky, viscous mucus layer where many of the resident bacteria are thought to live and that corals exude more of when stressed, significant autofluorescence (from fluorescent proteins produced by the coral and chlorophyll produced by the symbiotic dinoflagellates, zooxanthellae) that can overwhelm the signal from stains, and the size discrepancy between a coral polyp (millimeters to centimeters) and its microbial consortia that exist on the micrometer scale. Thus, the direct study of coral-microbe interactions at the scale most relevant to their ecological function, the microscale, has been challenging.

The microbial role in coral reef resilience to anthropogenic stress is a key issue that requires the elucidation of mechanisms at the microscale. There have been substantial discussions in the literature about how to define resilience (Nystrom et al 2008), how to manage reefs in ways that encourage it (Hughes et al 2010), and how to synthesize case studies to elucidate the large-scale mechanisms that determine whether a stony coral-dominated ecosystem can resist a phase-shift or rebound from one (Norstrom et al 2009). However, very little attention has been given to the small-scale mechanisms relevant to microbial processes that determine the resilience of individual corals to a given stressor. Delineating these mechanisms may be a critical link for understanding and predicting larger reef- and region-wide patterns of resilience.

Global coral reefs face a suite of anthropogenically-initiated stressors, ranging from decreasing pH of seawater and increasing sea surface temperatures to over-fishing and coastal development driven issues that include sedimentation, a variety of pollutants, and large influxes of excessive organic matter. Organic matter (OM) and nutrient pollution on reefs has been reviewed extensively (Fabricius 2005), and is a large problem for reefs all around the planet. When this dissertation work began, the ways in which reef microbes respond to OM enrichment were unknown.

We now know that coral-microbe relationships can be sensitive to organic matter inputs (Thurber et al 2009), and that there can be a surprising resilience at the single colony level on short time scales (Bourne et al 2008, Garren et al 2009). Elucidating the underlying mechanisms of this resilience remains a goal and would help to clarify how and why large-scale phase shifts occur on enriched reefs (i.e., what triggers the "tipping point").

Two sources of OM inputs are of particular growing concern for coral reefs: sewage outfalls and aquaculture. As the human population continues to grow, the need for sources of protein to eat and the need to dispose of waste also grow. Coastlines are already densely populated and aquaculture is the fastest growing animal-based food production sector on the planet (FAO 2006). Thus, understanding the mechanistic basis for coral-microbe responses to these sources of pollution are of prime interest with regard to conservation and management efforts.

It is from this context that the framework for this dissertation arose. It begins with a review of coral microbial ecology (Chapter 2) that highlights recent advances employing new technologies. In reviewing these studies, it becomes apparent that coral-microbe interactions are often studied in isolation of reef water and sediment microbial processes. There is a need to integrate our understanding of the microbial process that govern these different portions of the ecosystem and elucidate the mechanisms by which they occur at the scale on which they occur (the microscale). This chapter also provides a framework for thinking about organic matter on reefs as it naturally occurs, which is an important place to begin when considering how excess organic inputs may perturb microbial processes in time and space.

Chapter 3 describes a new method for quantifying bacteria in coral mucus. A critical first step toward microscale study of coral-associated bacteria is the fundamental need to see and quantify them reliably. The method is applied to a coral reef ecosystem influenced by sewage effluent to discover the surprising finding that corals exposed to enrichment do not have more bacteria in their mucus layer than reference corals, as we had predicted. Instead of influencing the number of bacteria present, the enrichment seemed to influence the size structure of the community. This suggests that

while the steady-state abundance of bacteria may be resilient to sewage effluent stress, the community does have a physiological response to that stress at a different level.

Chapter 4 focuses on the responses of reef-water microbial communities to OM inputs from coastal milkfish (Chanos chanos) pens in addition to examining coral-associated communities. The water-associated communities appeared less resilient to the OM inputs than coral-associated communities in that the abundance varied by more than 5-fold across the different water sites, and the community composition also varied substantially with distance from the fish pens. The percentage of the total water bacterial community attached to particles increased by more than 50-fold near the pens, suggesting physiological or life-strategy changes may be induced by such enrichment.

Chapter 5 describes a clonally replicated coral transplantation experiment at the same milkfish pens to examine the response of naïve coral-associated bacterial communities to high and low levels of pen effluent exposure over the time scale of days to weeks. We found that the communities on corals exposed to high levels of effluent were sensitive to this perturbation. They had drastically altered community compositions after five days and the abundance of bacteria in the coral mucus-tissue slurries were ~100-fold higher than communities from fragments at low effluent and reference sites. However, we also observed a surprising resilience of these communities. By the end of the experiment on day 22, the community composition and abundance at the high effluent sites both became similar to those of the corals at low effluent and reference sites.

Chapter 6 uses a combination of novel imaging technology (high speed laser scanning confocal microscopy on live coral and its natural associated bacterial community) and controlled aquaria experiments to investigate a microscale mechanism by which the resilience of coral-associated bacterial abundance we observed in previous chapters may occur. We tested the hypothesis that corals may release ("shed") bacteria into the surrounding water as a mechanism for controlling bacterial abundance on their surface. This is an idea that has been discussed for years because corals are known to release excess mucus in response to many types of stress, and it seems intuitive that bacteria may be released with that mucus. However, the technology to actually observe

such a phenomenon at the scale relevant to individual bacterial cells was not available until very recently. Here we observe this phenomenon in real time, and quantify an increase in the rate at which corals shed bacteria as a response to OM enrichment.

The dissertation concludes with some reflections on future directions for investigating the roles of microbes in coral reef resilience, and how such mechanisms might be elucidated at the microscale.

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Chapter 2

New directions in coral reef microbial ecology

Abstract

Microbial processes largely control the health and resilience of coral reef ecosystems, and new technologies have recently led to an exciting wave of discovery regarding the mechanisms by which microbial communities support the functioning of these incredibly diverse and valuable systems. There are three overarching questions currently at the forefront of discovery in coral reef microbiology. What are the mechanisms underlying coral reefs health and resilience? How do environmental and anthropogenic pressures affect reef ecosystem function? And what is the ecology of the microbial diseases of corals? The goal is to understand the functioning of coral reefs as integrated systems from microbes and molecules to regional and ocean-basin scale ecosystems to enable accurate predictions of ecosystem resilience and responses to environmental perturbations such as climate change and eutrophication. This review outlines recent discoveries in the microbial ecology of the different environments within coral reef ecosystems, and highlights research directions that can take advantage of new technologies to build a quantitative and mechanistic understanding of how holobiont health and resilience is connected to the microbial ecology of the environment in which it lives. The time is ripe for microbial ecologists to discover and create an integrated and mechanistic understanding of coral reef functioning. In the context of long-term survival and conservation of coral reef ecosystems, the need for this work is immediate.

Introduction

Microbial processes largely control the health and resilience of coral reef ecosystems, and new technologies have recently led to an exciting wave of discovery regarding the mechanisms by which microbes support the functioning of these incredibly diverse and societally valuable systems. Our current understanding is that a healthy coral reef is a finely tuned system that excels at capturing and recycling nutrients in oligotrophic waters and whose intricate three-dimensional structure allows for niche partitioning that supports astounding biodiversity and productivity. The individual reef-building corals embody this paradigm--animals living symbiotically with algae, viruses, bacteria,

archaea, and protists distributed in spatially distinct patterns to function as holobionts (Ainsworth et al 2010, Knowlton and Rohwer 2003). The great complexity of coral reef microbial ecology has until recently resisted functional, mechanistic and system-based analyses (Wild et al 2011). Novel molecular, biochemical and imaging advances have opened up the field and promise exciting fundamental discoveries. The time is ripe to deepen our understanding of these "captive" systems in intimate contact with the pelagic realm of the sea, and discover the mechanisms by which they exist as an integrated microbially supported system within the surrounding water and sediment environments.

There are three overarching questions currently at the forefront of discovery in coral reef microbiology. What are the mechanisms underlying coral reefs health and resilience? How do environmental and anthropogenic pressures affect reef ecosystem function? And what is the ecology of the microbial diseases of corals? The rapid decline of coral reefs globally creates a pressing need to answer these questions (Hughes et al 2010). The goal is to understand the functioning of coral reefs as integrated systems from microbes and molecules to regional and ocean-basin scale ecosystems to enable accurate predictions of ecosystem resilience and responses to environmental perturbations such as climate change and eutrophication.

The sediment, coral, and water environments of a reef are often studied in isolation of each other. A key component for answering all three of the driving questions is to uncover and quantify the mechanisms that tie these environments together into a single functioning ecosystem. For instance, we know that reef sediments generally have 10,000 times more bacteria than the surrounding seawaters (~10^9 cells cm^{-3} sediment vs. ~10^5 cells ml^{-1} seawater), and that coral-associated bacterial abundances are reported to range widely from approximately 1 x 10^2 to 6 x 10^7 cells per cm^2 (Coffroth 1990, Koren and Rosenberg 2006). However, the mechanisms within these different niches by which bacterial growth rates or community composition, for example, may aid in maintaining the stability and health of the coral holobiont have not yet been explored. Fortunately, new tools are now available that allow for a quantitative microbial ecology approach to discovering the mechanisms underlying these interactions and determining their relative roles in ecosystem

function. The goal of this review is to outline recent discoveries in the microbial ecology of the different environments within coral reef ecosystems, and to highlight research directions that will build a quantitative and mechanistic understanding of how holobiont health and resilience is connected to the microbial ecology of the environment in which it lives.

The Coral Holobiont

Reef-building corals are covered in a surface mucus layer composed of proteins, lipids, and polysaccharides (Brown and Bythell 2005, Tremblay et al 2011). Glycoproteins, in particular, are likely to be the mucus constituent responsible for its gelling properties and function (Jatkar et al 2010). The microbial communities inhabiting this surface region and the coral tissue below it have been studied more than in any other bacterial niche of the reef environment. Two recent articles (Ainsworth et al 2010, Mouchka et al 2010) offer an overview of the diversity and specificity of coral-bacteria associations, the onset of these associations (Sharp et al 2010), and many of the roles we currently understand microbes to play in coral health. These roles include subsidizing the host nutrient budget through nitrogen, carbon, and sulfur cycling, and assisting in disease resistance through the secretion of antimicrobial compounds to exclude colonization by exogenous bacteria. Additionally, Bourne and colleagues recently reviewed much of the research on coral diseases, and showed that while the ecology of a handful of pathogens has been elucidated, the study of most coral diseases will benefit from an integrated understanding of coral reef microbial ecology and access to new technologies and concepts from the biomedical field (Bourne et al 2009, Mao-Jones et al 2010). Thus, we will focus our discussion of the holobiont on discoveries made since the publication of those articles.

A number of recent advances have been made by applying the most current technologies available to the study of the holobiont. This exciting progress has opened new doors for mechanistically linking microbial ecology interactions within the holobiont to changes in the surrounding environments. For instance, pyrosequenceing has enabled the discovery of 17 metalloprotease genes in the genome of the coral pathogen *V. coralliityticus* that are potentially involved in its virulence (de O Santos et al 2011), as well as substantially higher levels of diversity

(Shannon Index of 6.71) and seasonal variability in bacterial community composition associated with a scleractinian coral than previously recognized (Chen et al 2011). Further, reef-associated algae have been identified as potential reservoirs of coral-disease associated bacteria (Barott et al 2011). Ultra-Performance Liquid Chromatography- Tandem Quad Mass Spectrometry (UPLC-MS/MS) was recently used to identify different responses in cyanotoxin production by several strains of black band disease (BBD)-associated cyanobacteria, often found to co-occur in an infection, to variations in environmental conditions (Stanic et al 2011). Quantitative and real-time polymerase chain reaction (qPCR and RT-PCR) techniques have also been applied to the coral holobiont to elucidate functional changes in the microbial members of the community over the course of disease progression (Bourne et al 2011). Uncovering the mechanisms of reef function with the aid of these new technologies is becoming ever more accessible, and the insights gained are now recognized as necessary for understanding the resilience of the ecosystems and developing effective conservation strategies.

Mucus as a Connection and Source of Structure

Within the holobiont, the mucus itself is an important energetic and ecological link between the coral animal and the surrounding water and sediment environments in which it lives (Mayer and Wild 2010, Naumann et al 2009, Rasheed et al 2004, Wild et al 2004a, Wild et al 2004b, Wild et al 2005). Mucus can play a role in structuring the coral-associated microbial communities while attached to the coral (Ritchie 2006), and once dissociated from the coral, can select for otherwise rare microbial members of the water community (Allers et al 2008). Microbial ecologists have proposed consideration of the marine environment as a size continuum of organic matter, ranging from truly dissolved organic carbon to large particles of detritus visible to the naked eye (Azam and Malfatti 2007). This concept can be applied to the corals themselves, which might be envisioned as essentially large particles covered with organic-rich mucus (Fig. 1).

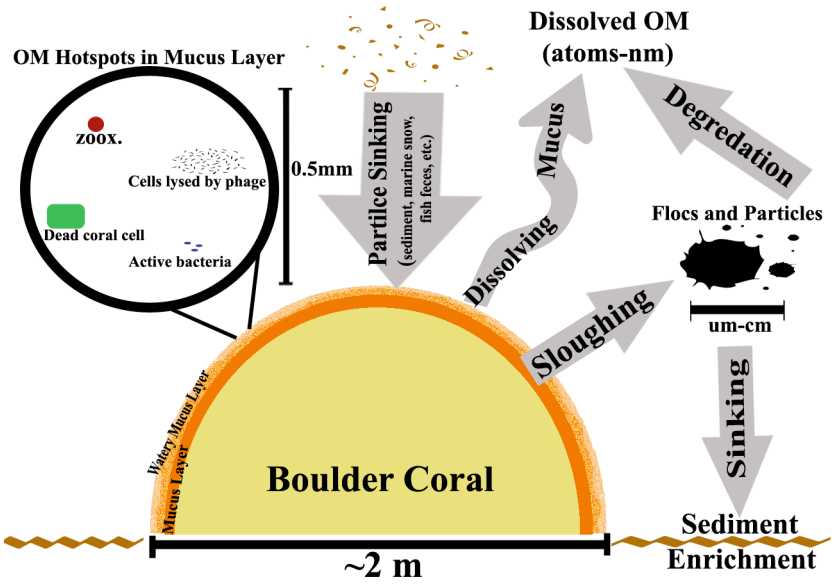

Figure 1-1. Coral in the context of the marine organic matter continuum (of both size and phase).

This framework allows the path of mucus to be followed as it morphs through a variety of microscale microbial habitats embedded in the organic matter continuum, connecting the microbial communities of the holobiont to those in the surrounding seawater and the sediments, and providing a variety of physical architectures for microbial interaction. For instance, as mucus sloughs off from the coral into the surrounding water it can dissolve (van Duyl and Gast 2001) (potentially fueling water column microbial production), floc (to be eaten by larger detritivores or degraded by microbes), or sink (to be consumed by sediment microbial communities) (Huettel et al 2006, Mayer and Wild 2010, Naumann et al 2009, Vacelet and Thomassin 1991, Wild et al 2006) (Fig. 1). In the context of studying the mechanisms of health and resilience of coral reefs, quantifying the magnitude and variability of these fluxes, and how they respond to perturbation is a major research subject. Clarifying the role of microscale architecture in these mechanisms is also important: if the spatial distribution of organic matter in the water column changes, would the distribution of coral-associated microbiota also change?

Coral Reef Waters

Researchers have explored the microbial processes occurring in a variety of reef water habitats—everywhere from remote atoll lagoons (Rochelle-Newall et al 2008, Sorokin 1978,

Torreton and Dufour 1996a, Torreton and Dufour 1996b, Torreton et al 2000, Yoshinaga et al 1991) to the water overlaying heavily anthropogenically influenced coastal reefs (Garren et al 2008, Gast et al 1999, Hoch et al 2008, van Duyl et al 2002). A common theme that appears across these diverse habitats is that the benthos influences the water column environment, and it does so at several different scales.

At the scale of individual colonies, a coral influences the niche structure for microbes at the millimeter to centimeter scale. Corals can release large quantities of dissolved organic carbon (DOC; 2-25 μM (mg protein)$^{-1}$ d^{-l}) and dissolved organic nitrogen (DON; 0.5-3 μM (mg protein)$^{-1}$ d^{-l}) (Ferrier-Pages et al 1998, Naumann et al 2010), and water in contact with the coral surface can be enriched in labile DOC, have significantly higher bacterial specific growth rates than surrounding water, and have elevated oxygen concentrations compared to water overlaying algal-dominated substrate (Tanaka et al 2011, van Duyl and Gast 2001, Wild et al 2010). Some coral species have been shown to consume bacterioplankton directly as a source of phosphorus, nitrogen and up to 20% of their carbon demand (Johannes et al 1972, Sorokin 1973a, Sorokin 1973b). This is consistent with observations that cavities in the reef framework can have 29% less bacterioplankton and 15% less DOC than surround reef water (de Goeij and van Duyl 2007). Further, 12 cm long transects from the surface of corals have shown an average of a 2-fold increase in bacteria abundance and a 3.5-fold increase in virus-like particle (VLP) abundance in the 4 cm of water nearest to the surface (as compared to the abundances 8-12 cm away) (Seymour et al 2005). High DNA (HDNA) bacteria cells, a measure of the proportion of actively dividing cells, were also most abundant in the 4 cm closest to the coral surface (Seymour et al 2005) and were 10% higher above live coral surfaces than in water 1 m away despite the fact that total bacterial abundances were lower (Patten et al 2006). The physiological state of the coral colony can influence these small-scale habitats. For instance, VLPs can be 30% more abundant in the 12 cm above diseased corals compared with healthy and dead colonies (Patten et al 2006).

At the larger scale of a single reef, phytoplankton and bacteria abundances in the water column can be depleted by the filter feeding action of the benthos (Gast et al 1999, Genin et al 2009,

Linley and Koop 1986, Yahel et al 1998, Yahel et al 2006) and the size structure of phytoplankton communities can be shifted toward larger cells by size-selective filter feeding (van Duyl et al 2002). The concentrations of DOC and bacterial cells can become depleted over the scale of several kilometers even on a rapidly flushed reef (Nelson et al 2011). Phytoplankton, bacteria, and virus blooms can all be stimulated by coral mass spawning events (Patten et al 2008, Wild et al 2008) as can changes in bacterial community composition (Apprill and Rappe 2011). The physical oceanography of a reef can also drastically influence the observed nutrient levels and fluxes. An internal tidal bore, for instance, can increase nutrient concentrations 10-40 fold relative to non-bore conditions (Leichter et al 2003).

Evidence suggests that nitrogen metabolism and production rates of bacterioplankton in reef waters are sensitive to eutrophication (Hoch et al 2008). However, in general, the large-scale mechanisms controlling nutrient fluxes and their relationship to microscale processes that may be critical for individual coral health remain to be identified. For instance, it would be useful to know if there are microscale hotspots of nutrient cycling and regeneration that influence the large-scale patterns we observe or if spatial heterogeneity plays a role in regulating the flux of nutrients from the water column into the coral holobiont, perhaps by the action of coral-associated bacteria and archaea.

Reef Sediments

In shallow reef habitats, the water column is usually well mixed and sediment resuspension can shape the microbial seascape and create interaction opportunities among the benthic, water column, and coral microbial communities (Rasheed et al 2004, Yahel et al 2002). Reef sediments are generally well colonized (on the order of 1 to 2 x 10^9 cells cm^{-2} (Wild et al 2006)) by a diverse community of microbes (Gaidos et al 2011, Hewson and Fuhrman 2006, Pringault et al 2008, Rusch et al 2009). The microbial communities in reef sediments play an important role in benthic-pelagic coupling through the degradation of sloughed coral mucus and nitrogen cycling (Gaidos et al 2011). This mucus traps organic particles, bacteria, and picoplankton in the water column, and eventually settles on the sediments where up to 7% can be degraded per hour (Huettel et al 2006, Mayer and Wild 2010, Naumann et al 2009, Wild et al 2004b). At Heron Island (Great Barrier Reef, Australia),

researchers found that the total production from benthic diatoms, dinoflagellates, and cyanobacteria in the sediments is of the same order of magnitude as the production by corals themselves (Werner et al 2006), and that those sediments were both net calcifying and nitrogen fixing.

A significant amount of nitrogen is thought to be exported from calcareous reef sediments to the rest of the ecosystem through grazing and resuspension of benthic microalgae that readily take up N fixed in the sediments (Miyajima et al 2001). All benthic environments, such as sand, coral rubble, live coral, and cyanobacterial mats on coral reefs that have been examined were found to be active in N_2-fixation (Casareto et al 2008, Charpy et al 2010, Larkum et al 1988, Ohba et al 2009, Shashar et al 1994). For example, the sandy bottom of a lagoon in the Red Sea provided 70% of the nitrogen fixation to the surrounding fringing reef (Shashar et al 1994) and endolithic algae living in coral rubble generated enough nitrogen to support up to 28% of primary production on the reef at La Reunion Island in the Indian Ocean (Casareto et al 2008).

It has been shown that anthropogenic enrichment of reef sediments, in this case from fish farms, can saturate the ability of the microbial communities to metabolize organic material, and shift the sediment environment to one that is dominated entirely by anaerobic metabolism (Holmer et al 2003). The spatial resolution of microscale niches within the sediments has rarely been examined, and thus our mechanistic understanding of the coupling between benthic and pelagic processes remains at a larger, coarser scale than is needed for accurate predictions of ecosystem responses to perturbation.

One example of a specific mechanism that allows interaction among water column, coral mucus, and sediment microbial communities is the process of mucus bundle formation in response to sedimentation (Smriga 2010). When sediment is deposited on some coral species, such as *Montipora aequituberculata* and *Acropora microphthalma*, mucus is exuded that entrains the sediment and is released from the coral. These bundles then behave as particles in the water column that can continue to entrain other particles or plankton, be consumed by detritivores, or sink onto the benthos (perhaps another coral, an alga, or sediment). This is just one example of the tight coupling that can exist among the various reef microscale habitats. The grazing and subsequent defecation patterns of

mobile reef organisms (such as fish and invertebrates) provide another such mechanism (Johannes et al 1972, Smriga et al 2010).

In thinking about the various connections among the microbial habitats discussed thus far, it is important to consider the time component and the role sediments might play as an archive of anthropogenic influence. For instance, Cu and Zn contamination in reef sediments can reach high levels from a single ship grounding (Jones 2007, Negri et al 2002) such that small amounts of the sediment are toxic to coral larvae and inhibit settlement (Negri et al 2002, Smith et al 2003). The selection pressure of these metals can shift sediment bacterial community composition, and increase the incidence of antibiotic resistance (Nogales et al 2011). This metal signature may persist in the sediments and/or the associated bacterial communities for many years to come with unexplored consequences for overall ecosystem function. This is an example of why we need to understand the propagation of sublethal perturbations through the ecosystem to be able to predict the responses and resilience of coral reefs to future change.

Toward a Mechanistic and Quantitative Understanding of Reef Microbial Ecology

Given the strong coupling in coral reef ecosystems, it is not possible to clearly separate benthic and pelagic processes, and thus we need to understand the holobiont within the continuum of benthic and pelagic environments. In a similar context, each of these environments encompasses many different microscale niches that can change the dynamics of benthic-pelagic interactions depending on the physical processes and various types of external forcing at play. In the pelagic environment, for example, we know that organic matter aggregates such as marine snow and phytoplankton are point sources of high concentrations of organic matter that vary on the millimeter scale, and are profoundly important for the functioning of ocean basin scale pelagic marine ecosystems (Azam 1998, Kiorboe and Jackson 2001, Long and Azam 2001, Seymour et al 2000). Similarly, we can consider how microscale architecture and its heterogeneous distribution may regulate some mechanisms of microbial interactions. Spatial heterogeneity within coral microbial communities has been documented on the scale of centimeters (Rohwer et al 2001), but has rarely been investigated on a smaller scale. For example, one potential mechanism to consider is local

organic matter enrichment within the coral mucus layer by expelled zooxanthellae (Baghdasarian and Muscatine 2000, Garren and Azam 2010, Jones and Yellowlees 1997, Paul et al 1986, Wild et al 2005), which could act as a hotspot for microbial growth, as in the case of rapid and profuse colonization by *Vibrio cholerae* of the marine dinoflagellate *Lingulodinium polyedrum* (Mueller et al 2007). Heterogeneity in the microscale physical architecture of the coral mucus layer may contribute to spatial variation in microbial interactions such as bacteria-bacteria antagonism (Ritchie 2006, Rypien et al 2010), nitrogen cycling (Beman et al 2007, Olson et al 2009, Siboni et al 2008), or pathogen survival (Looney et al 2010).

A parallel continuum of size must be layered into our understanding as organic matter, the fuel for microbial processes, is considered within the spatial continuum of the reef environment. One of the next challenges for the field is to elucidate and quantify the mechanistic connections among these microbial communities within this multidimensional, multi-scale framework to attain a cause-and-effect understanding of how a reef ecosystem functions. The technology is now available to begin adding multiple spatial and time scale contexts to fundamental questions of coral reef ecosystem function such as how growth of associated microbial communities is regulated, why we see certain dominances in a given niche, what the primary sources of bacterial mortality are under specific environmental conditions, and how all of these mechanisms relate to the health or disease state of a reef (See Box 1). For example, new developments in confocal imaging technology have enabled the visualization of the natural coral-microbial assemblage in situ, and micro-scale ecological interactions can now be observed in real time (Garren and Azam, in prep).

As we consider questions that are currently driving the field of coral microbial ecology forward, it is an opportune moment to reflect on the progress that has been made in recent history. In 2003, Knowlton and Rohwer wrote an article in The American Naturalist entitled *Multispecies Microbial Mutualisms on Coral Reefs: Host as Habitat*. They outlined the then most current understanding of bacterial and archaeal associations with corals, and concluded that "we know almost nothing about the role of noneukaryotic microbes in healthy coral" (p.S54). Among the research questions that they suggested to drive the field forward were: 1) what is the scope of

diversity for coral-associated bacteria and archaea, and how is it patterned in space and time? 2) Which of the many bacterial and archaeal associates of corals are true mutualists, and what roles do they play? 3) How important are bacterial and archaeal communities to the health of coral reefs, and are they being disrupted by anthropogenic stress?

The field has made substantial progress on these questions in the past eight years, and yet there remains much to be learned by revisiting these same questions and taking advantage of significant advances in genomics and imagining technologies. As discussed earlier and recently reviewed by others (Ainsworth et al 2010, Bourne et al 2009, Mouchka et al 2010), the scope of diversity of bacteria is much clearer than it was before, and we have more information about patterns of distribution and diversity through space and time. However, the same cannot be said for the archaeal communities, and there remain many levels of spatial and time scales on which we do not understand the bacterial behaviors. Further, and critically, our understanding of *in situ* microbial activity and interactions is still quite incomplete. It has become clear that studying the tropical reef environment requires some modifications of methods from traditional temperate marine microbial ecology to accurately quantify essential parameters (e.g. (Torreton and Dufour 1996a) for FDDC translation into μ, (Garren and Azam 2010, Wild et al 2006) for enumeration of bacterial cells), and these breakthroughs are helping to constrain and quantify critical pieces of the puzzle such as bacterial abundance, growth rates, and production rates (Table 1).

Table 1. Reported values showing the range of measurements for bacterial abundance, growth rates, and primary production made in coral reef environments.

Sample Type	Location	Bacteria Abundance (x 10^5 cells/ml)	Bacterial Growth Rate (μ; day^{-1})	Primary Production	Growth and Production Methods	Source
Epilithic and Endolithic Cyanobacteria on Coral Rubble	Sesoko Island, Okinawa, Japan (May 2007)	-	-	134.6 ± 55.2 nmoles C μg Chl-a^{-1} day^{-1}	^{13}C Tracer following Hama et al 1993	(Casareto et al 2008)
Reef Water (overlying, crevice, and bottom)	Curacao	3 to 8	0.12 to 0.48	-	Tritiated Leucine incorp.	(Gast et al 1998)
Reef Water	Florida Keys, USA	4	1.1± 0.2	24.0 ± 4.8 μg C L^{-1} day^{-1}	^{14}C HCO^-_3; ^{14}C leucine	(Hoch et al 2008)
Atoll Lagoon Water	Tuamotu Archipelago, French Polynesia	6 (Takapoto) to 18 (Tikehau)	0.72 to 4.01	-	Tritiated Thymidine incorp.	(Torreton and Dufour 1996b)
Reef Water	Barrier Reef, Belize	3.5	0.53	1.1 μg C L^{-1} day^{-1} (cyanobacteria only)	Seawater culture technique	(Herndl 1991)
Atoll Lagoon Water	12 Atolls in the Tuamotu Archipelago, French Polynesia	2.2 (Tekokota)to 20.7 (Hiti)	0.07 (Marokau) to 1.54 (Reka-Reka)	1 (Tekokota) to 91 (Reka-Reka) μg C L^{-1} day^{-1}	Tritiated Thymidine incorp.; ^{14}C HCO^-_3	(Torreton et al 2002)
Reef Waters around remote atolls	Northern Line Islands	0.72 (Kingman) to 8.4 (Kiritimati)	-	-	-	(Dinsdale et al 2008)

Table 1 continued.

Sample Type	Location	Bacteria Abundance (x 10^5 cells/ml)	Bacterial Growth Rate (µ; day^{-1})	Primary Production	Growth and Production Methods	Source
Reef Waters (near fish farms)	Bolinao, Philippines	15.1 to 74.3	0.23 to 0.26	-	Frequency of dividing-divided cells (Conversion to µ following Torreton & Dufour 1996)	(Garren et al 2009)
Reef Waters	Marshall, Gilbert, & Hawaiian Islands; Great Barrier Reef	9.5 (Majura Atoll) to 39.5 (Butaritari Atoll)	0.30 (Butaritari) to 0.65 (Kaneohe Bay, Oahu)	4.1 (Majura Atoll) to 67 (Heron Island, GBR) µg C L^{-1} day^{-1}	^{14}C labeling for both bacteria and phytoplankton production	(Sorokin 1973a)
Atoll Lagoon Water	Majero Atoll	8 to 14	0.24 to 0.85	-	Tritiated thymidine & tritiated leucine incorp.	(Yoshinaga et al 1991)
Reef Waters	New Caledonia	5.5 to 7.4	0.07 to 0.34	5.6 to 12.2 µg C L^{-1} day^{-1}	Tritiated Thymidine incorp.; ^{14}C HCO^-_3	(Torreton et al 2010)

And thus, while the questions we highlight (Box 1) address some of the current gaps in our understanding of reef microbial ecology, many are questions that have been asked previously. The difference in asking them now is that new technologies (including NanoSIMS, super resolution microscopy, high-speed imaging techniques, confocal Raman microspectroscopy, next-generation sequencing, and microfluidics) are becoming accessible to marine microbial ecologists that can help answer them in a quantitative way. In this new era of microbial ecology, some researchers are considering phage therapy as a potential response to global increases in coral disease (Efrony et al 2007, Efrony et al 2009) and the ability to employ the rapidly advancing concepts and techniques from biomedicine is ever more accessible (Bourne et al 2009). Studies of coral reef ecology and microbial diseases of corals would also benefit by learning from the rapidly advancing field of

human health considerations of microbial pathogenesis and the ecology of microbial diseases. Indeed, future discoveries in coral-microbe interactions may "return the favors" through potential applicability to human-microbe interactions. The time is ripe for microbial ecologists to discover and create an integrated and mechanistic understanding of coral reef functioning. In the context of long-term survival and conservation of coral reef ecosystems, the need for this work is immediate.

Box 1: Questions to Pursue

Health and Resilience

1. What is a "healthy coral reef ecosystem" from a microbial ecology perspective? What are its limits of resilience?
2. What are the magnitude and variability of biogeochemical fluxes, and what are their responses to perturbation (e.g., pH, eutrophication, etc.)?
3. Do the water or sediment ecosystems buffer corals from environmental change? How much perturbation can the water column or sediment microbial communities mediate such that corals remain relatively unaffected by nutrient inputs or heavy metal contamination?
4. How important are the relative growth rates and community compositions of bacteria and archaea in different microniches to the stability, health, and resilience of the ecosystem?

Disease Ecology

5. Are there microbes that can act as early warning signs of imminent decline or disturbance? (e.g., a μ-canary in the coral mine, so to speak)
6. How frequently are microbes exchanged from one microenvironment to another and what mechanisms mediate this exchange? What are the transport mechanisms?
7. Do rare phylotypes have the potential to become dominant if they reach a new habitat?

Anthropogenic Influences

8. What role can anthropogenic influences play in tipping the balance from rare to abundant for certain phylotypes?
9. If the spatial distribution of organic matter in the water column changes, does the distribution of coral-associated microbiota also change?
10. How do sublethal perturbations propagate through the ecosystem?

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Chapter 3

New method for counting bacteria associated with coral mucus

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New Method for Counting Bacteria Associated with Coral Mucus[▽]†

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The ability to count bacteria associated with reef-building corals in a rapid, reliable, and cost-effective manner has been hindered by the viscous and highly autofluorescent nature of the coral mucus layer (CML) in which they live. We present a new method that disperses bacterial cells by trypsinization prior to 4′,6-diamidino-2-phenylindole (DAPI) staining and quantification by epifluorescence microscopy. We sampled seawater and coral mucus from *Porites lobata* from 6 reef sites influenced by wastewater intrusion and 2 reef sites unaffected by wastewater in Hawaii. Bacterial and zooxanthella abundances and cell sizes were quantified for each sample. Bacteria were more abundant in coral mucus (ranging from $5.3 \times 10^5 \pm 1.0 \times 10^5$ cells ml^{-1} to $1.8 \times 10^6 \pm 0.2 \times 10^6$ cells ml^{-1}) than in the surrounding seawater ($1.9 \times 10^5 \pm 0.1 \times 10^5$ cells ml^{-1} to $4.2 \times 10^5 \pm 0.2 \times 10^5$ cells ml^{-1}), and the mucus-associated cells were significantly smaller than their seawater counterparts at all sites ($P < 0.0001$). The difference in cell size between mucus- and seawater-associated bacteria decreased at wastewater-influenced sites, where simultaneously mucus bacteria were larger and seawater bacteria were smaller than those at uninfluenced sites. The abundance of zooxanthellae in mucus ranged from $1.1 \times 10^5 \pm 0.1 \times 10^5$ cells ml^{-1} to $3.4 \times 10^5 \pm 0.3 \times 10^5$ cells ml^{-1}. The frequency of dividing cells (FDC) was higher in the surrounding seawater than in mucus, despite finding that a 1,000-fold-higher zooxanthella biovolume than bacterial biovolume existed in the CML. Establishment of a standardized protocol for enumeration will provide the field of coral microbial ecology with the urgently needed ability to compare observations across studies and regions.

The extremely viscous and highly autofluorescent nature of coral mucus has been a major challenge in developing enumeration techniques and has limited our ability to study the ecological interactions among coral mucus layer (CML)-associated microbial communities. Only a few studies have used direct counts to quantify bacteria in the CML, and the methods and subsequent results vary widely. The techniques have included scanning electron microscopy (SEM) (34), phase-contrast microscopy (27), and epifluorescent microscopy using a variety of stains (acridine orange staining [8], SYBR gold [20], and 4′,6-diamidino-2-phenylindole [DAPI] [3]). Bacterial abundances reported from these studies spanned more than 5 orders of magnitude (from 1.6×10^2 cells [cm^2]$^{-1}$ using acridine orange [8] to 6.2×10^7 cells [cm^2]$^{-1}$ using SYBR gold [20]), and some of the studies are difficult to compare to each other because different units were used, such as cells ml^{-1} of mucus and cells (cm^2)$^{-1}$ of coral. Some variation in abundance is likely due to differences in mucus sampling methods and differences among coral species. However, the enormous quantity of autofluorescence emitted in green and red wavelengths found in most coral species creates a substantial challenge for reliably counting fluorescently stained cells in that portion of the spectrum, because many of the particles are bacterium sized. Many of these same particles could be visible with phase-contrast microscopy as well. Thus far, researchers quantifying CML-associated bacteria using epifluorescence microscopy have prepared their samples by following well-established protocols that were developed for seawater. We suggest that the viscous and autofluorescent nature of coral mucus may require some modifications from standard seawater protocols for epifluorescence microscopy to be most effective.

SEM is an alternative to fluorescence-dependent techniques. It has the advantage of acquiring images with sufficient detail to distinguish among particles and cells, but this method is time-consuming, visualizes only the surface of the sample, and is not widely available or affordable enough for it to be a standard field protocol. An additional limitation is that most studies that have employed SEM for CML observation have found bacteria to be too dispersed to count in a reasonable number of micrographs (8, 19).

Here we present a new method that disperses bacterial cells by enzymatically digesting the mucus with trypsin (an adaptation of routine cellular biology cell line culture procedures) and subsequently staining the cells with DAPI for rapid quantification using epifluorescence microscopy. DAPI fluoresces in the blue end of the spectrum, and its emission does not overlap with the autofluorescence of the mucus samples. This method is rapid, uses reagents and equipment readily available in microbial ecology laboratories, and can provide necessary information for studies of the ecology of microbial cells associated with mucus. It may also be helpful for studies of other aquatic gel-associated microbial communities.

This visualization capability revealed that bacteria living with the reef-building coral *Porites lobata* were significantly smaller than their water-associated counterparts and that this difference is reduced in reefs heavily influenced by anthropogenic impacts. There is only one other report that we are aware of that observed small bacterial cell size in mucus from corals

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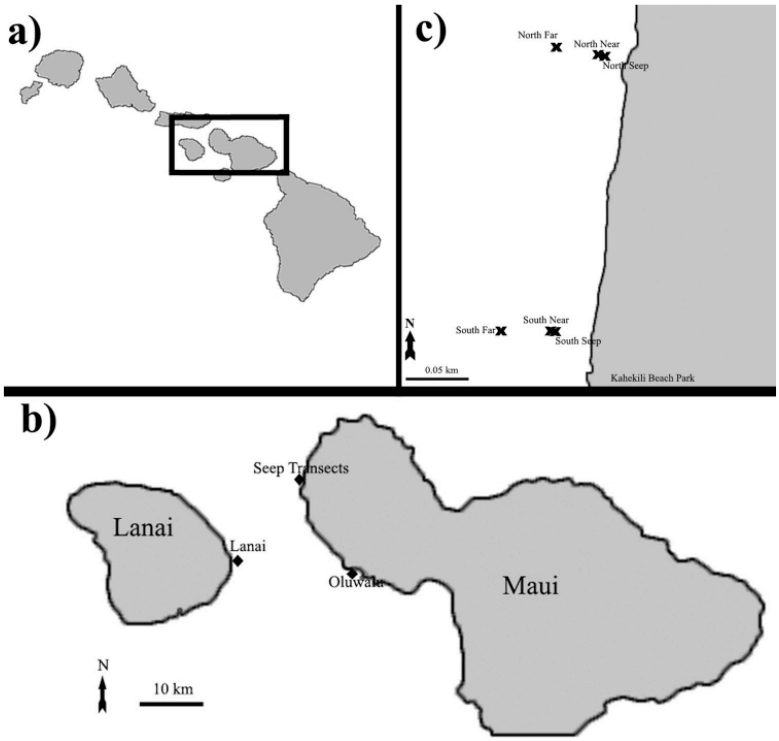

FIG. 1. (a) Overview of the Hawaiian Islands. (b) Our 3 major sampling locations on Maui and Lanai. (c) Close-up view of the two wastewater-influenced transects.

(of the genus *Fungia*), but that study did not quantify cell size (34). Given that mucus is a carbon-rich environment (6, 11, 12, 18, 24, 25, 31), this discovery is counterintuitive. It highlights questions regarding the ecological interactions that must occur *in situ* to select for small cell size in such a rich environment (3, 4, 7, 8, 11, 25, 34).

MATERIALS AND METHODS

Sampling sites. In July 2009, samples were collected from 7 different sites on the west side of Maui, HI, and 1 site on the east side of Lanai, HI (Fig. 1). There were two transects (north and south; 3 sites each) that began at freshwater seeps known to carry wastewater treatment plant effluent to the reef (10). On both transects, no living coral existed directly at the seep sites, so the "near" sites were chosen as the closest living coral cover offshore of the seep (~2 m away from each seep), and the "far" sites were ~50 m west (offshore) of the near sites. All 6 sites (seep, near, and far sites for both the north and south transects) showed the influence of wastewater in nitrogen isotope fractionation throughout the year (10). The two control sites that did not show evidence of wastewater influence were at Oluwalu and Lanai, HI (Fig. 1) (10). Samples were also collected from flowthrough seawater aquaria maintained at Scripps Institution of Oceanography (SIO), La Jolla, CA, for comparison.

Sample collection. Triplicate samples were collected by scuba diving at each site of seawater 0.5 m above the substrate (using 4-liter Cubitainers) and of coral mucus from *Porites lobata* (using 10-ml syringes) all between 1 to 5 m in depth. The two exceptions were at the seep sites, where only seawater was collected because no live coral cover existed. Samples were kept at ambient seawater temperature in the dark until arrival at the lab (<1 h), where they were fixed with 2% 0.02-μm-filtered formaldehyde. The mucus samples formed a compact mucus ball in the syringe, and that ball was removed with a sterile pipette tip, rinsed briefly in autoclaved 0.02-μm-filtered seawater to removed loosely associated seawater bacteria, and then fixed. All samples were allowed to fix at 4°C for at least 4 h. Mucus samples were stored frozen without filtering, while water samples were filtered on 0.2-μm polycarbonate filters (17), dried, wrapped in foil, and stored at −20°C until processing.

For the coral species *Acropora yongei*, maintained in aquaria at SIO, two mucus collection methods were compared. Triplicate mucus samples were collected using a 10-ml syringe, as done in the field, and an additional set of samples were collected by quickly rinsing the coral with 0.02-μm-filtered autoclaved seawater (FASW) to remove any loosely associated bacteria and then exposing the fragments to air for 3 min over a sterile 1.5-ml tube. Triplicate seawater samples were collected from the tank using sterile 15-ml Falcon tubes from the inflow valve and from directly above the coral samples. All samples were fixed as described above.

Seawater carryover experiment. To quantify the number of water-associated bacteria remaining in coral mucus samples, *A. yongei* fragments were sampled in triplicate using the two methods described above (air exposure and syringe). A total of 200 μl of each mucus sample was removed with a cut, sterile 1,000-μl tip and put into 1 ml of FASW with 2% 0.02-μm-filtered formaldehyde. (Cut tips were used to reduce physical sheering of the viscous sample during transfer.) Samples were gently inverted three times and incubated at room temperature for 10 min to allow any loosely associated cells to disassociate. Samples were spun for 45 s at 4,000 × *g* to gently pellet the mucus. A total of 1 ml of supernatant was removed, fixed at 4°C overnight, and filtered onto 0.2-μm polycarbonate filters to enumerate (see below) as "carryover," while the mucus pellets had another 2% 0.02-μm-filtered formaldehyde added to them before being put at 4°C overnight. Mucus samples were then treated and enumerated by following the trypsin protocol described below. Tank seawater was collected (as described above) and enumerated (as described below) at the same time, and FASW controls were carried along as well.

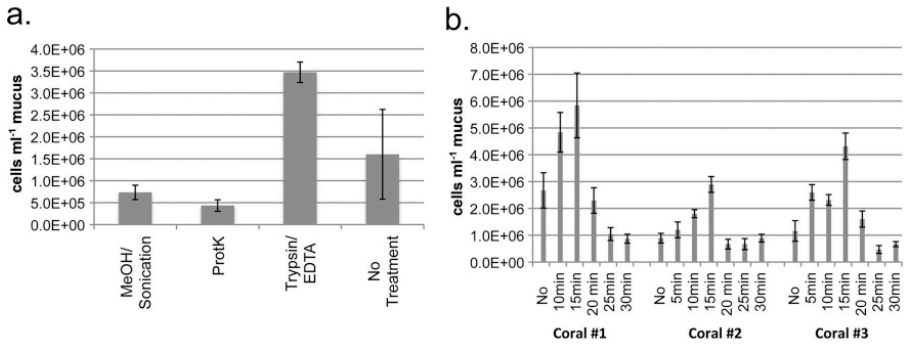

FIG. 2. (a) Average bacterial cell counts obtained from 3 *P. lobata* mucus samples, either untreated ("No Treatment") or treated with methanol and sonication, subjected to proteinase K digest, or treated with EDTA followed by 10 min of trypsin incubation. (b) Bacterial cell counts obtained from *P. lobata* mucus treated with 30 min of EDTA incubation followed by varied trypsin incubation durations.

Mucus-associated bacterial dispersal treatment experiments. Fixed mucus from three *Porites lobata* colonies was used for dispersal experiments. No-treatment controls for each colony were included in each trial. Three different methods were initially tried on 1 ml of 1:10 dilutions of mucus in 0.02 μm filtered autoclaved Milli-Q water, as follows: (i) 10% methanol incubated at 35°C for 15 min and sonicated on ice for three rounds of 1 min at 50% power and 30 s rest (adapted from reference 22), (ii) 20 μg ml^{-1} proteinase K digestion at room temperature for 10 min (adapted from reference 3), and (iii) 10 μM EDTA incubation on ice, followed by 0.4% trypsin incubation for 10 min at 37°C (quenched on ice) (adapted from reference 26). All samples (including untreated controls) were incubated for 8 min at 4°C with 5 μg ml^{-1} DAPI before being filtered onto black 0.2-μm polycarbonate filters. Filters were dried in the dark, wrapped in foil, and stored at −20°C until prepared for counting (see below).

To find the best incubation length for 0.4% trypsin at 37°C, the subsamples of mucus from each of the three corals were incubated for 0, 5, 10, 15, 20, 25, and 30 min (see the supplemental material for additional optimization experiments). All other parameters were maintained as described above. The following three different protocols were tried after the 15 min of trypsin incubation to quench autofluorescence present in the mucus samples: (i) 2 mg ml^{-1} of crystal violet was added at the DAPI staining step (adapted from reference 15), (ii) 500 μl 1× acidic Alcian blue was incubated on the filter (after DAPI staining) while the sample was in the filter tower for 45 s before filtering through (adapted from reference 2), and (iii) 1 mg ml^{-1} $FeSO_4$ incubated on ice for 30 min before DAPI staining (adapted from reference 1). Three trials examined the effect of prefiltration after trypsin treatment with 10-μm-, 3-μm-, and 1-μm-pore-size filters on the abundances of bacteria, zooxanthellae, and autofluorescent particles in subsequent mucus samples collected on 0.2-μm polycarbonate filters (17).

Dispersal procedure. The final procedure used for obtaining all results presented after the trials described above was dilution of fixed mucus at 1:10 in 0.02 μm filtered autoclaved Milli-Q water using a cut, sterile 1-ml tip, vortexing for 10 s at maximum speed, incubation for 30 min on ice with 10 μM EDTA (to chelate cations that inhibit the enzymatic activity of trypsin), addition of 0.4% trypsin, incubation for 15 min at 37°C, quenching on the ice-water slurry, addition of 5 μg ml^{-1} DAPI, incubation for 8 min in the dark at 4°C, immediate filtering onto a 0.2-μm black polycarbonate filter, drying in the dark, and store wrapping in foil at −20°C until slide preparation.

Counting and sizing of cells. Stored DAPI-stained 0.2-μm filters were thawed, and one-quarter of each filter was mounted onto glass slides using Vectashield mounting medium (Vector Labs, CA) mixed with 2 μg ml^{-1} of the lipid stain 10-*N*-nonyl-acridine orange (NAO; Sigma-Aldrich, St. Louis, MO), while the rest of the filter was archived at −20°C. NAO provides a secondary stain to confirm the DAPI counts. Only dual-stained DAPI-NAO cells were enumerated. Eubacterial fluorescence *in situ* hybridization (FISH) probes were also used to confirm DAPI counts (Cy5-labeled EUB-388 I to III and a non-EUB-388 control) (protocol adapted from reference 13). Zooxanthellae were counted at 400× magnification, and bacterial cells and the frequency of dividing cells (FDC) (14) were counted at 1,000× magnification on a Nikon Eclipse TE-2000U. Images were taken using the NIS Elements software program. The dimensions of 100 to 200 bacterial cells and 20 zooxanthella cells were measured from each sample using the NIS-Elements measurement function in the DAPI channel for bacteria and the tetramethyl rhodamine isocyanate (TRITC) channel for zooxanthellae. Biovolume was estimated by the formula $V = (\pi/4)W^2 \times (L - W/3)$, where L is the length and W is the width (5). DAPI, FITC, TRITC, and 5-cyano-2,3-ditoyl tetrazolium chloride (CTC) filter sets were used to quantify fluorescent particles from the quenching trials. To verify the abundances counted with the epifluorescence microscope, two other microscopy techniques were employed. The same slides were enumerated using laser scanning confocal microscopy *z*-stacks (Nikon Eclipse TE-2000U) to include the full *z*-axis dimension of each sample. Scanning electron microscopy (SEM) was done by following an established protocol (34) for half of the archived portion of filters.

Statistics. The software program JMP was used for statistical analysis of cell length data. Cell lengths from water and coral mucus bacteria at all sites were transformed using Box-Cox analysis and tested for normality using a Shapiro-Wilk test. Analysis of variance (ANOVA) was performed to quantify the effect of "site" and "source" (cells from coral mucus or water) on cell length. A post hoc Tukey-Kramer honest significant difference (HSD) test was performed to determine which sets of sites were significantly different from each other for each source.

RESULTS

Cell counting methods. Our initial attempts to adapt methanol, sonication, and proteinase K protocols yielded lower counts than the no-treatment control protocol (Fig. 2a). Dissociation of cells with a trypsin digestion was more successful than use of other treatments and the no-treatment controls. The error in counting samples from *P. lobata* was reduced by more than 9-fold compared to that in untreated samples when digested with trypsin, while total cell counts were >2.5 times higher in trypsin-treated *A. yongei* samples than in untreated samples. We found that 15 min of 0.4% trypsin incubation maximized the number of DAPI-stained cells visualized by epifluorescent microscopy (Fig. 2b). Attempts to quench autofluorescent particles in the coral mucus were marginally successful (see Fig. S1 in the supplemental material); however, these treatments also reduced our ability to count zooxanthella cells (see Fig. S2 in the supplemental material). Prefiltration experiments to physically remove autofluorescent particles after trypsin treatment reduced final bacterial cell counts by a minimum of 2-fold (with a 10-μm prefilter) or removed almost all cells (3-μm and 1-μm prefilters). DAPI cell counts were successfully verified with a secondary label using both NAO and Eub388 fluorescence *in situ* hybridization (FISH) probes.

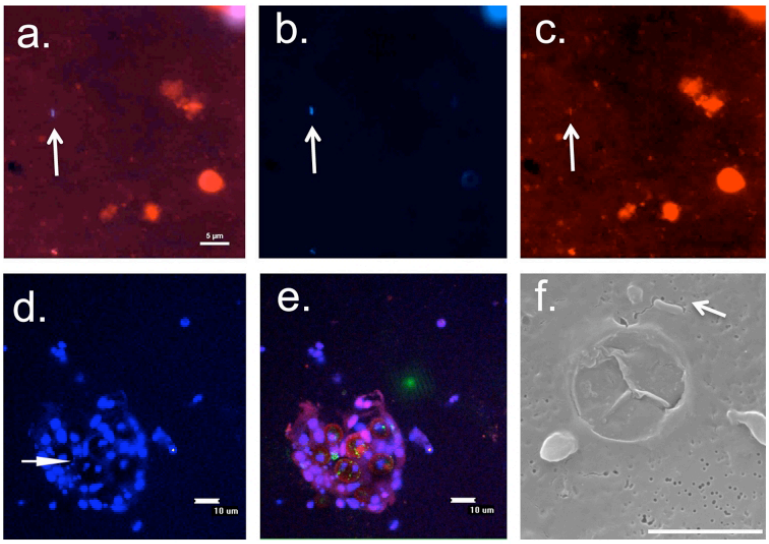

FIG. 3. Representative images of *P. lobata* mucus treated with the trypsin protocol. (a to c) Single images of a mucus sample, each with an arrow pointing toward a bacterial cell (the scale bar is 5 μm). Epifluorescence images of a dual-stained sample showing red (NAO and chlorophyll) and blue (DAPI) channels overlaid (a), the blue channel alone (b), and the red channel alone (c). (d and e) Integrated three-dimensional stacks displayed in two dimensions of a single field of view taken with laser scanning confocal microscopy, with an arrow pointing toward several bacterial cells (the scale bar is 10 μm). (d) Blue channel shown from a laser scanning confocal image, showing the DAPI signal. The arrow points toward bacterial cells in a cluster of zooxanthella cells. (e) Red, green, and blue channels from the same image as shown in panel d, showing the autofluorescence detected in the red and green channels. (f) SEM image of a bacterium (arrow) in the mucus, with a 5-μm scale bar.

The final dispersal procedure is described above but, in short, involved 15 min of trypsin incubation, no quenching of autofluorescence, 5 μg ml^{-1} DAPI staining, filtration, and mounting the slide with Vectashield mixed with NAO to stain lipids as a secondary stain—only dual NAO-DAPI-stained cells were enumerated (Fig. 3a to c).

The intrusion level of bacteria from surrounding seawater was low with both the syringe and air exposure mucus collection methods (see Fig. S3 in the supplemental material). Air-exposed samples carried over 3.9 to 6.8% of total cell counts from entrained seawater in the mucus, while syringe-collected mucus brought in 5.8 to 11.5% of total counts. These percentages are within the error of any given count.

Abundances. Bacterial abundances in *P. lobata* mucus ranged from $5.3 \times 10^5 \pm 1.0 \times 10^5$ cells ml^{-1} at Oluwalu to $1.8 \times 10^6 \pm 0.2 \times 10^6$ cells ml^{-1} at the north near site (Table 1). The surrounding seawater at each site had concentrations ranging from $1.9 \times 10^5 \pm 0.1 \times 10^5$ cells ml^{-1} at Oluwalu to $4.1 \times 10^5 \pm 0.2 \times 10^5$ cells ml^{-1} at the south near site (Table 1). Zooxanthella cell abundance in *P. lobata* mucus ranged from $1.1 \times 10^5 \pm 0.1 \times 10^5$ cells ml^{-1} at the north far site to $3.4 \times 10^5 \pm 0.3 \times 10^5$ cells ml^{-1} at Oluwalu (Table 1). The frequency of dividing cells (FDC) was higher in the surrounding seawater than in the coral mucus (Table 1).

The bacterial abundances assessed by laser scanning confocal microscopy using *z*-stacks to image the full three dimen-

TABLE 1. Seawater and coral mucus cell data from all sites[a]

Site	Bacterial abundance (10^5 cells ml^{-1})		Zooxanthella abundance in mucus (10^5 cells ml^{-1})	Bacterial FDC (% of DAPI count)		Avg cell length of bacteria (μm)	
	Mucus	Seawater		Mucus	Seawater	Mucus	Seawater
Lanai	13.6 ± 0.2	3.3 ± 0.1	1.1 ± 0.2	3	7	0.36 ± 0.01	0.78 ± 0.04
Oluwalu	5.3 ± 0.1	1.9 ± 0.1	3.4 ± 0.3	4	11	0.44 ± 0.02	0.86 ± 0.03
North far	15.3 ± 0.3	2.6 ± 0.1	1.1 ± 0.1	7	6	0.49 ± 0.02	0.62 ± 0.02
North near	13.8 ± 0.3	4.1 ± 0.2	1.4 ± 0.1	5	12	0.52 ± 0.02	0.73 ± 0.03
North seep	NA	3.4 ± 0.3	NA	NA	12	NA	0.67 ± 0.02
South far	12.8 ± 0.2	3.0 ± 0.1	2.3 ± 0.2	6	9	0.54 ± 0.02	0.76 ± 0.03
South near	17.9 ± 0.2	3.6 ± 0.1	1.8 ± 0.2	5	11	0.58 ± 0.02	0.75 ± 0.03
South seep	NA	3.8 ± 0.1	NA	NA	11	NA	0.69 ± 0.02

[a] NA, not applicable.

sions of each sample were the same, within error, as the epifluorescence counts for both water and trypsin-treated coral mucus samples (Fig. 3d and e). Tenfold fewer bacterial cells (a maximum of 3.7×10^5 cells ml^{-1}) were observed by scanning electron microscopy (SEM) than by either of the other methods in *P. lobata* mucus (Fig. 3f).

Cell size. Bacterial cells from *P. lobata* mucus were significantly ($P < 0.0001$) shorter in length than their seawater counterparts at every site (Table 1). Seawater bacteria were 2.0 to 2.2 times longer than mucus-associated bacteria at sites uninfluenced by wastewater effluent (Lanai and Oluwalu) and 1.3 to 1.4 times longer at effluent-receiving sites (all north and south sites). The smallest length measured for any zooxanthella cell was 6.92 μm, which is more than 12 times longer than the longest average cell length from any mucus-associated bacterial community (0.58 μm; south near site). Bacterial cells associated with *P. lobata* mucus had an average volume of $5.2 \times 10^{-2} \pm 0.6 \times 10^{-2}$ μm^3, while zooxanthella cells were more than 4 orders of magnitude larger, with an average volume of $8.7 \times 10^2 \pm 3.0 \times 10^2$ μm^3.

DISCUSSION

Digestion of the mucus sample before filtration dramatically improved our ability to visualize and enumerate coral-associated bacterial cells. Trypsin is a serine protease that specifically cleaves peptide chains on the carboxyl side of lysine or arginine. Hydrolysis is slowed if an acidic residue is on either side of the cleavage site, and cleavage does not occur at all if a proline residue is located on the C-terminal side of the site (9). These characteristics contribute to the variation in trypsin sensitivity that has been observed among different cell lines (29). In the case of marine bacteria, we observed at least one culture that appears insensitive to trypsinization and two distinct natural assemblages (CML associated and seawater associated) that had different levels of sensitivity (see the supplemental material). The protocol we have proposed here may be useful for microbial ecology studies of any aquatic gel-associated community, but the trypsinization step should be optimized for each new type of sample. Our results provide a practical starting place for designing optimization experiments for other marine samples.

Observed cell counts are always considered to be a minimum estimate of actual abundance. The two main things that we wanted to verify during the development of this method were (i) that all "cells" counted were indeed actual cells and (ii) that the sample preparation sufficiently dispersed the cells so that they could be reliably counted in two dimensions using epifluorescence microscopy. To address the first concern, we used eubacterial FISH probes as well as a lipid stain (NAO) to confirm the DAPI counts. The difference between DAPI-only cell abundance and dual-stained cell abundance was within the errors of the counts (<2.5%). In the interest of creating a streamlined protocol, we used the equally effective NAO dual-staining method in place of FISH for routine counting. Unstained mucus samples did not fluoresce in the blue channel, which also supported the use of DAPI rather than other stains.

We used laser scanning confocal microscopy and SEM to address the second point. Confocal microscopy has the advantage of being able to optically slice the sample and create three-dimensional images that include the insides of potentially thick mucus particles. The fact that confocal counts were within errors (±<2.5%) of epifluorescence counts suggests that the trypsin treatment and subsequent volume of mucus filtered sufficiently dispersed the sample such that very few cells were out of the primary plane of focus. The disadvantage of confocal microscopy is that it remains a fluorescence-dependent technique. SEM can image cell shape independently of fluorescence but lacks the ability to see beyond the surface of the sample. The fact that we generally observed 10-fold fewer cells with SEM than with fluorescence-based techniques, by following an established protocol (34), could be due to cell loss during preparation or gold-palladium sputtering that obscured the morphology of cells still embedded in a thin layer of mucus. It is also possible that some of the particles we counted were, in fact, not cells. However, given that both FISH probes and a lipid stain confirmed the DAPI cell counts, it is unlikely that this is the case. Our observations are in accord with two other studies that found that SEM underestimated bacteria associated with coral mucus (8, 19).

Two general sources of potential error in coral-associated bacterial counts are as follows: (i) missed cells that were too deeply entrained in mucus to either be stained or be visualized and (ii) the incorrect enumeration of bacterium-sized autofluorescent particles in green and red areas of the spectrum. We observed an average of $9.3 \times 10^6 \pm 0.7 \times 10^6$ green (FITC) particles per milliliter of untreated mucus in *P. lobata* samples, while average DAPI-stained bacterial concentrations were never greater than $1.8 \times 10^6 \pm 0.2 \times 10^6$ cells ml^{-1} of mucus. If a stain with emission in the green portion of the spectrum was used, such as SYBR green I or acridine orange, bacterial abundance would have been overestimated by at least 1 order of magnitude.

We found that mucus collection by either air exposure or syringe suction is appropriate. The bacterial cell abundances we observed are within the range of other reported values. Some degree of species-to-species variability is expected (as we observed between *A. yongei* and *P. lobata*), and thus, standardization of a method becomes even more important. Given that there are less than 10 published studies (3, 8, 16, 21, 27, 28, 34) reporting directly counted bacterial concentrations in the mucus layer, and none of those studies examined the same species of coral from the same geographic region nor used the same methods, we are at the very early stages of acquiring a baseline as a field. This is an ideal time to develop a standard method on which all subsequent studies can build. This protocol is a useful starting point in the development of a toolbox of consistent methods that will allow microbial ecology hypotheses to be tested in the viscous, autofluorescent mucus environment.

This is among the first reports of zooxanthella concentrations in coral mucus. They were far more abundant in *P. lobata* than expected. Cell biovolume measurements show that zooxanthellae are more than 4 orders of magnitude larger than bacteria. These cell volumes are likely an underestimate of the true volume, due to shrinking that occurs during fixation (see reference 23 for a full discussion). Zooxanthella abundance was only 1 order of magnitude less than bacterial abundance, indicating that 1,000-fold more algal biomass existed in the mucus layer than bacterial biomass. Given that dinoflagellates tend to exude photosynthate, and bacteria readily grow using that carbon source (3, 11, 31), why are mucus-associated bacteria so much smaller than their seawater counterparts?

The opposite might be expected in an organically enriched environment. The higher frequency of dividing cells (FDC) in surrounding seawater than in mucus may suggest that CML bacteria are not capitalizing on enrichment from zooxanthella exudate for growth or that the zooxanthellae are less "leaky" than other dinoflagellates. It is also possible that the CML environment is distinct enough from the surrounding seawater that the FDC cannot be used to directly compare the bacterial growth rates between these two communities. Torreton and Dufour (33) found that the frequency of dividing-divided cells (FDDC) in tropical reef waters could not be directly interpreted in terms of growth rates of the assemblage using temperate water conversion factors (14) without severely overestimating bacterial production (by comparing the FDDC-based calculations to thymidine and leucine incorporation rates in the same samples). Those authors reinforce the necessity of calibrating FDC and FDDC conversion factors when working in any new ecosystem. It is possible that in the CML, the conversion factors for the FDC to bacterial production would be significantly different from the surrounding seawater.

Another question raised by this study concerns why wastewater effluent would encourage seawater-associated bacteria to be smaller than cells on uninfluenced reefs. The observation of significantly larger and more abundant mucus-associated bacteria at wastewater-influenced sites ($P < 0.0001$) could be explained by excess surrounding nutrients stimulating growth or by a higher proportion of water-associated bacteria invading into the mucus layer. Perhaps mucus bacteria are generally small due to size-selective grazing pressure in the CML (by coral polyps or other microbes), antagonistic interactions among bacteria (30, 32), or a limiting nutrient or micronutrient (thus encouraging a higher surface area/volume ratio). While our study cannot answer these questions, the described method for visualizing and enumerating bacteria should assist in future resolution of such questions for coral microbial ecology.

ACKNOWLEDGMENTS

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Supplemental Material

Method for Additional Trypsinization Optimization Experiments

To understand the sharp peak in CML bacteria cell counts with a 15 min digestion at 37°C, *P. lobata* mucus was treated as above while changing only the trypsin incubation. Three separate incubations were performed on aliquots of the same mucus: 1) 0.4% trypsin incubated at room temperature (~22°C) for 10 min, 30 min, 60 min, and 120 min. 2) 0.4% trypsin incubated at 37°C for 13 min, 15 min, 16 min, 17 min, and 19 min. 3) 0.2% trypsin incubated at 37°C for 10 min, 15 min, 20 min, 30 min, and 60 min. Fixed seawater samples from the Scripps Pier, La Jolla, CA were filtered onto 0.2 μm polycarbonate filters, rinsed with sterile MilliQ water, and incubated with 100 μl 0.4% trypsin at 37°C for 0 min, 10 min, 15 min, 20 min, and 60 min. A broth culture of the marine isolate SWAT 3 (R. A. Long, D. C. Rowley, E. Zamora, J. Y. Liu, D. H. Bartlett, and F. Azam, Applied and Environmental Microbiology **71**:8531-8536, 2005) was fixed with 2% 0.2 μm filtered formaldehyde, diluted 1:100 in sterile MilliQ, incubated with 10 μM EDTA and 0.4% trypsin at 37°C for 0 min, 5 min, 10 min, 15 min, 30 min, and 90 min. All samples were stained with DAPI, mounted onto slides with VectaShield mounting medium (Vector Labs, California, USA), and counted as described in main text.

Results from Additional Trypsinization Optimization Experiments

All treatments on coral mucus showed a similar curve of increasing abundance of visible cells to a certain incubation length, and then the cell count decreased with longer incubations (Fig. S4). Incubating at room temperature increased the amount of time that the samples could be trypsinized to 60 minutes. Using half the amount of trypsin (0.2% instead of 0.4%) slowed the process considerably and created a longer time window in which samples could be stopped; however, the maximum total abundance was lower than the 0.4% trypsin treatments. The 0.4% incubation at 37°C was effective, but created a short window of 15-16 minutes in which the reaction had to be stopped before cell counts dropped sharply. The abundance of the marine isolate SWAT3 did not decrease

significantly with 0.4% trypsinization even after 90 minutes. The number of visible cells in the natural seawater assemblage decreased by ~29% from the 15 minute incubation to the 60 minute incubation (0.4% trypsinization at 37°C; 1.95± 0.11 x 10^6 cells ml^{-1} vs. 1.38 ± 0.08 x 10^6 cells ml^{-1}).

Supplemental Figures

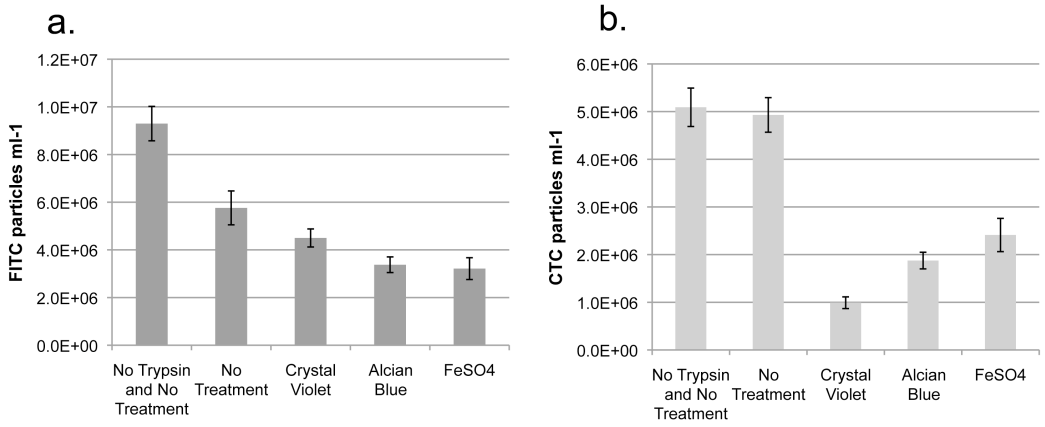

Figure S1. Trials to reduce autofluorescence of mucus samples prior to DAPI staining. a) The number of particles counted using FITC filters. b) The number of particles per ml observed using CTC filters.

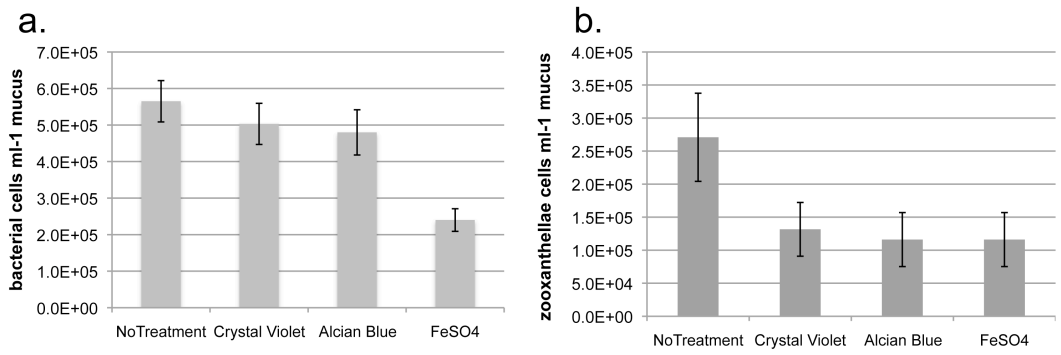

Figure S2. a) Average bacteria counts from mucus samples treated with EDTA/trypsin and one of 4 treatments (none, crystal violet, acidic alcian blue, or $FeSO_4$) to quench autofluorescence before DAPI staining. b) Zooxanthellae abundances from the same samples shown in (a).

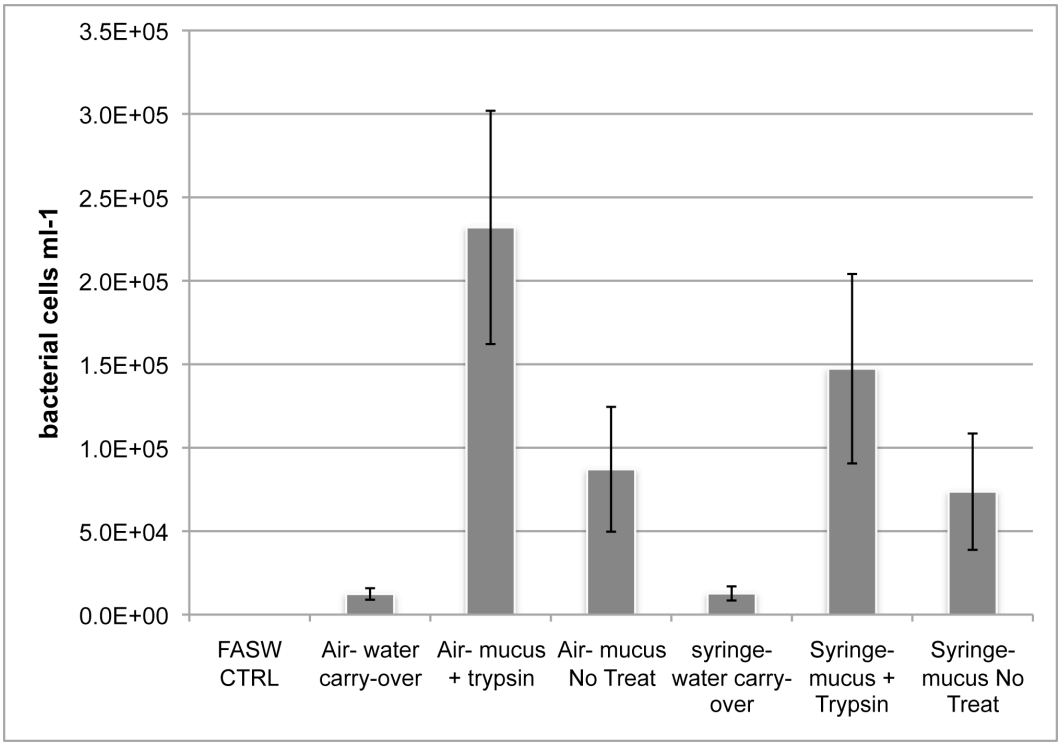

Figure S3. The number of bacteria enumerated in *A. youngii* mucus (with and without trypsinization) and the water carried along during mucus sampling using two separate collection methods (air-exposure and syringe). The 0.02 μm filtered autoclaved seawater (FASW) used to dilute the mucus samples was quantified as a control.

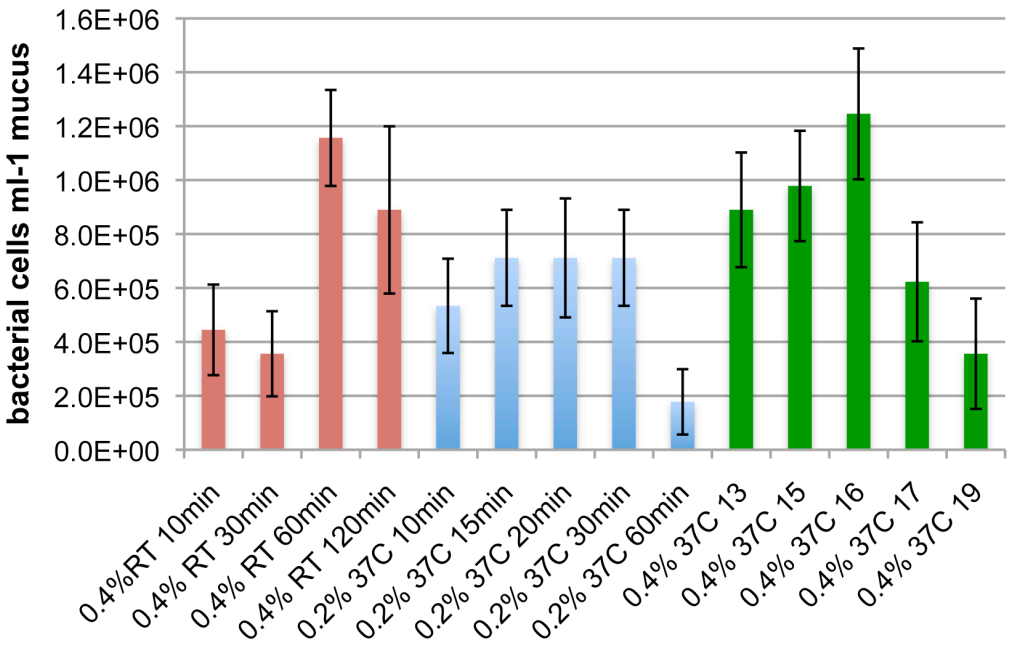

Figure S4. Three separate experiments were performed on aliquots of the same mucus samples. The results of a 0.4% trypsin incubation at room temperature for 10, 30, 60, and 120 minutes are shown in red. Blue bars represent the abundances for the 0.2% trypsin incubated at 37°C for 10, 15, 20, 30, and 90 minutes, while the green bars represent the 0.4% trypsin incubated at 37°C for 13, 15, 16,17, and 19 minutes.

Chapter 3 is a full reprint of the publication (including supplemental materials that were published online-only): M. Garren and F. Azam (2010) A New Method for Counting Bacteria Associated with Coral Mucus. *Applied and Environmental Microbiology* 76: 6128-6133, with permission from the coauthor.

Chapter 4

Gradients of coastal fish farm effluents and their effect on coral reef microbes

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Gradients of coastal fish farm effluents and their effect on coral reef microbes

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Summary

Coastal milkfish (*Chanos chanos*) farming may be a source of organic matter enrichment for coral reefs in Bolinao, Republic of the Philippines. Interactions among microbial communities associated with the water column, corals and milkfish feces can provide insight into the ecosystem's response to enrichment. Samples were collected at sites along a transect that extended from suspended milkfish pens into the coral reef. Water was characterized by steep gradients in the concentrations of dissolved organic carbon (70–160 μM), total dissolved nitrogen (7–40 μM), chlorophyll *a* (0.25–10 μg l^{-1}), particulate matter (106–832 μg l^{-1}), bacteria (5×10^5–1×10^6 cells ml^{-1}) and viruses ($1–7 \times 10^7$ ml^{-1}) that correlated with distance from the fish cages. Particle-attached bacteria, which were observed by scanning laser confocal microscopy, increased across the gradient from < 0.1% to 5.6% of total bacteria at the fish pens. Analyses of 16S rRNA genes by denaturing gradient gel electrophoresis and environmental clone libraries revealed distinct microbial communities for each sample type. Coral libraries had the greatest number of phyla represented (range: 6–8) while fish feces contained the lowest number (3). Coral libraries also had the greatest number of 'novel' sequences (defined as < 93% similar to any sequence in the NCBI nt database; 29% compared with 3% and 5% in the feces and seawater libraries respectively). Despite the differences in microbial community composition, some 16S rRNA sequences co-occurred across sample types including *Acinetobacter* sp. and *Ralstonia* sp. Such patterns raise the question of whether bacteria might be transported from the fish pens to corals or if microenvironments at the fish pens and on the corals select for the same phylotypes. Understanding the underlying mechanisms of effluent–coral interactions will help predict the ability of coral reef ecosystems to resist and rebound from organic matter enrichment.

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Location of Study: Bolinao, Pangasinan, Republic of the Philippines.

Introduction

Microbes drive biogeochemical cycles in coastal ecosystems (Kirchman *et al*., 2007) including coral reefs (Torreton and Dufour, 1996a,b; Van Duyl and Gast, 2001). They regulate carbon fluxes (reviewed in Azam and Malfatti, 2007), fix and remineralize nutrients, and regulate inorganic nutrient fluxes (Kirchman, 1994; Bidle and Azam, 1999; Moran *et al*., 2003). Mesocosm experiments have shown that enrichment of coastal waters can cause trophic cascades among plankton communities, enable bacteria to out-compete phytoplankton for nutrients, and change the net flux of particulate organic carbon (POC) depending on the type of enrichment applied (Joint *et al*., 2002; Havskum *et al*., 2003; Hasegawa *et al*., 2005; Davidson *et al*., 2007). Bacteria abundances in mesocosm experiments can increase dramatically when coastal water is enriched with dissolved material from an intense phytoplankton bloom (Joint *et al*., 2002; Worden *et al*., 2006) or when coral reef water is enriched with various carbon sources (Mitchell and Chet, 1975). Increased microbial abundances are also associated with human sewage effluents in coastal coral reef waters (Paul *et al*., 1997; Gast *et al*., 1999).

Within the context of microbial contributions to biogeochemical dynamics, there is a nuanced set of interactions occurring between specific microbial taxa and the distinct components of the marine environment with which they consistently associate. For example, *Cytophaga* gene sequences were associated with high-molecular-weight dissolved organic matter (e.g. Cottrell and Kirchman, 2000) and particles (Riemann *et al*., 2000). Some *Roseobacters* associate with phytoplankton (Gonzalez *et al*., 2000, Buchan *et al*., 2005) while some *Betaproteobacteria* may associate with diatom blooms (Morris *et al*., 2006). An epidemiologically relevant association is that of *Vibrio cholerae* with marine copepods (Huq *et al*., 1983; Reviewed in Grimes, 1991). Understanding the ecological roles of marine microenvironment-specific microbial associations

Table 1. The chemical characteristics of particulate and dissolved phases of water collected from each site. Average values are presented (±SE).

Site	DOC (μM)	Total nitrogen (dissolved, μM)	Chl *a* (μg l^{-1})	Phaeophytin (μg l^{-1})	Chl:Pha	POC (μg l^{-1})	Total nitrogen (particulate, μg l^{-1})
A	69.7 ± 1.3	7.4 ± 0.4	0.25 ± 0.03	0.26 ± 0.05	0.98	106 ± 4	9 ± 15
B	80.4 ± 2.9	8.0 ± 0.2	0.28 ± 0.03	0.20 ± 0.03	1.39	196 ± 57	39 ± 15
C	89.6 ± 1.7	14.2 ± 0.7	0.38 ± 0.03	0.50 ± 0.07	0.76	662 ± 68	54 ± 17
D	141 ± 2.9	30.5 ± 1.3	4.5 ± 0.2	3.04 ± 0.1	1.47	832 ± 338	86 ± 45
Fish pens	162 ± 18.5	39.8 ± 2.7	10.3 ± 0.2	3.84 ± 0.2	2.69	641 ± 60	86 ± 18

can lend insight into large-scale ecological processes and help elucidate the mechanisms of ecosystem-wide responses to nutrient perturbations. Microbial connections may exist among several marine microenvironments within a tropical coral reef ecosystem, and the influence of nutrient enrichment on such connections may be large for coral reefs adjacent to coastal fish farms.

Fish farms may stress nearby coral reefs and lead to ecosystem degradation and decreased resilience (Hughes and Connel, 1999), like other localized activities such as fishing (Pandolfi *et al.*, 2003; Bascompte *et al.*, 2005), human sewage inputs (reviewed in Fabricius, 2005) and sedimentation from land development (reviewed in Fabricius, 2005). Marine fish farms disperse particulate organic matter into underlying sediments (Holmer and Kristensen, 1992), enrich surrounding waters with nutrients (Hall *et al.*, 1990; Enell, 1995; Loya *et al.*, 2004), and change the composition of sediment macrofaunal communities (Karakassis *et al.*, 2000). Among the few studies that have directly addressed interactions between tropical fish farms and coral reefs, nutrients released from gilthead sea bream (*Sparus aurata*) farms adversely affected larvae production in corals (Loya *et al.*, 2004) and at least one coral species, *Pocillopora damicornis*, experienced low survivorship along a gradient of milkfish cage effluents (Villanueva *et al.*, 2005).

Intensive milkfish mariculture is one of several environmental stressors that may impact coral reefs near Bolinao, Pangasinan province, Republic of the Philippines. In addition to the low coral survivorship observed by Villanueva and colleagues (2005), other studies in the region found that benthic sediments near suspended milkfish cages are enriched with organic and inorganic compounds. The amount of enrichment inversely correlates with distance from the cages (Holmer *et al.*, 2002; 2003). Both sedimentation rates and sediment oxygen consumption rates were elevated in the cages (Holmer *et al.*, 2002; 2003). While macrofauna and sediments have been considered within the fish farm-coral reef environment, the roles of microbial communities in this context remain poorly understood. The effects of effluents on coral-associated microbial communities may provide a mechanistic link between milkfish farms and coral reefs. To explore this relationship, the following hypotheses were tested in Bolinao: (i) milkfish farms influence the chemical and physical characteristics of waters in the adjacent coral reef ecosystem; (ii) physical characteristics of the waters are correlated with differences in microbial assemblages within the reef; and (iii) distinct microbial groups found in fish farms co-occur in water and corals.

Results

Nutrients and chlorophyll a

Total dissolved nitrogen (TN) and dissolved organic carbon (DOC) concentrations in the water column were negatively correlated with distance from the fish pens ($P < 0.0001$). Sites A and B were not significantly different from each other. Total dissolved nitrogen values ranged from 6.8 to 8.3 μM and DOC from 68.0 to 84.4 μM. Site C is significantly different in TN concentration ($P = 0.0392$, 12.9–15.0 μM), but not in DOC concentration (86.2–91.6 μM) from Sites A and B. Similarly, Site D and the fish pen site are significantly different from Sites A–C ($P < 0.0001$) in both TN and DOC concentrations, and are significantly different from each other in TN concentration. The DOC concentration ranged from 138.3 to 198.2 μM and the TN concentration was 28.7–33.0 μM at Site D and 37.0–45.2 μM at the pens. Sites A and B had approximately threefold less POC than Sites C, D and fish pens. Chlorophyll *a* concentrations ranged between 0.25 and 0.38 μg l^{-1} (phaeophytin 0.20–0.50 μg l^{-1}) at Sites A–C. Site D and the fish pens were significantly different from each other and all other sites ($P < 0.0001$) with 4.5 μg l^{-1} and 10.3 μg l^{-1} respectively (phaeophytin 3.04 and 3.84 μg l^{-1} respectively; Table 1).

Abundances of bacteria, particles and viruses

Free-living bacteria and total bacteria (free-living plus particle-attached) average abundances were not significantly different among Sites A, B and D (Fig. 1A; Table 2). Mean abundances for these sites ranged from 4.1 to 6.1×10^5 free-living bacteria ml^{-1} ($4.1–6.1 \times 10^5$ total bacteria ml^{-1}). Site C had significantly lower abundances of bacteria ($P = 0.0033$; 3.0×10^5 free-living bacteria ml^{-1}

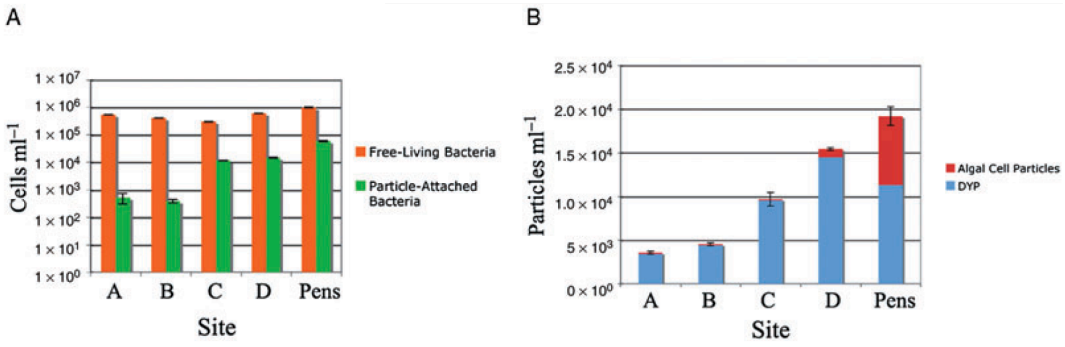

Fig. 1. A. Abundance of free-living and particle-attached bacteria in the water column.
B. Total particle abundance separated into the two categories of particles quantified: algal cell-derived and DAPI stained yellow particles (DYP). All error bars represent standard error. Data also presented in Table 2.

and 3.1×10^5 total bacteria ml^{-1}) while the fish pens had significantly higher abundances than all other sites ($P = 0.0006$; 9.9×10^5 free-living bacteria ml^{-1} and 1.0×10^6 total bacteria ml^{-1}). The mean abundance of particle-attached bacteria per ml ranged from 5.3×10^2 at Site A to 5.8×10^4 at the fish pens. Particles at Sites A–C and the fish pens are similarly colonized on average (0.01–0.06 bacteria cells μm^{-2}, Fig. 2) while Site D had generally less colonized particles (0.005 bacteria cells μm^{-2}). Particle-attached bacteria represented less than 0.1% of total bacteria at Sites A and B, 3.7% at Site C, 2.3% at Site D, and 5.6% at the fish pens (Fig. 3A; Table 2).

Total particle abundance correlates with the inverse of distance to the fish pens ($P = 0.0038$; Fig. 1B). Total particle abundance ranged from 1.18 to 6.39×10^3 ml^{-1} while DYP abundance ranged from 3.39×10^3–1.44×10^4 ml^{-1}. Mean particle size also increased near the fish pens from 20 to 65 μm^2 per particle at Sites A–C to 281–576 μm^2 per particle at Sites D and fish pens. Virus-like particles (VLP) increase across the Sites towards the fish pens from 1.0 to 7.0×10^7 ml^{-1} (Fig. 3B; Table 2).

Microbial community composition

Denaturing gradient gel electrophoresis (DGGE) showed distinct microbial communities in each sample type. The basic banding pattern was the same across all sites within the particle-attached or the free-living water column communities. However, there were several bands within each sample type that were only present at Sites A–C or only at Sites C, D and the fish pens (Fig. 2). The three major phyla represented among the 27 water sample DGGE bands that were cloned and sequenced included *Proteobacteria* (44%), *Cyanobacteria* (22%) and *Bacteroidetes* (15%). Of the 17 comigrating pairs of DGGE bands found at the same position on the gel that were cloned and sequenced, nine pairs came from bands originating from two different sample types (i.e. free-living water fraction, particle-attached water fraction, coral slurry or feces) and eight pairs came from bands of samples within the same sample type. Six of the nine pairs from different sample types were not of the same phyla (examples can be seen in Fig. 2; 2A band 1/3P band1, 2A

Table 2. Abundances of microbes and particles (DAPI yellow particles and algal cells) from water sampled at each site.

Site	Virus-like particles abundance (number ml^{-1})	Free-living bacteria abundance[a] (cells ml^{-1})	Particle-attached bacteria abundance[a] (cells ml^{-1})	% of total bacteria attached to particles	# of particles per ml[a]		Average particle size (μm^2)
					DYP	Algal	
A	$1.0 \pm 0.07 \times 10^7$	$5.4 \pm 0.3 \times 10^5$	$5.3 \pm 2.2 \times 10^2$	< 0.1	$3.4 \pm 0.2 \times 10^3$	$1.6 \pm 0.2 \times 10^2$	42.7
B	$0.8 \pm 0.04 \times 10^7$	$4.2 \pm 0.6 \times 10^5$	$3.9 \pm 0.6 \times 10^2$	< 0.1	$4.4 \pm 0.2 \times 10^3$	$1.0 \pm 0.1 \times 10^2$	19.7
C	$1.7 \pm 0.1 \times 10^7$	$3.0 \pm 0.04 \times 10^5$	$113.7 \pm 3.6 \times 10^2$	3.7	$9.6 \pm 0.8 \times 10^3$	$1.1 \pm 0.1 \times 10^2$	65.8
D	$7.0 \pm 0.3 \times 10^7$	$6.1 \pm 0.6 \times 10^5$	$144.5 \pm 5.6 \times 10^2$	2.3	$14.4 \pm 0.1 \times 10^3$	$9.7 \pm 0.7 \times 10^2$	576.1
Fish pens	$6.1 \pm 0.7 \times 10^7$	$9.9 \pm 0.3 \times 10^5$	$583.2 \pm 28.1 \times 10^2$	5.6	$11.3 \pm 0.5 \times 10^3$	$78.4 \pm 5.5 \times 10^2$	280.8

a. Data from Fig. 5.
Average values are presented (±SE).

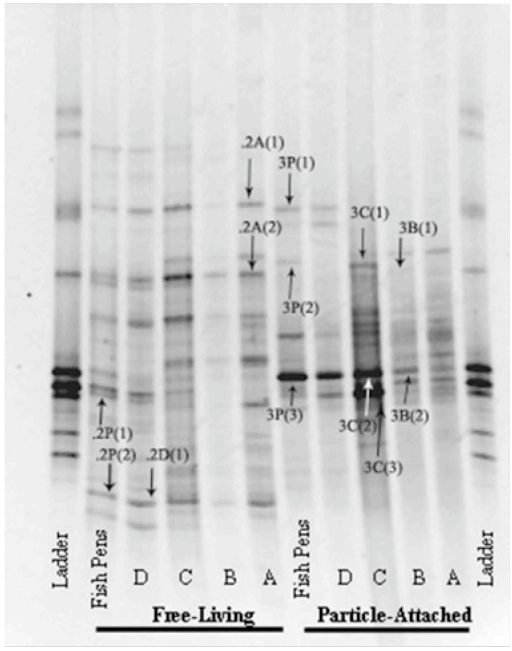

Fig. 2. DGGE banding profiles (V3 region of 16S rRNA gene) of water from each site revealed distinct free-living and particle-attached communities as well as a few phylotypes that appeared near the pens [e.g. 3P(1), 2P(2), 2D(1)]. Each lane contains pooled PCR products from triplicate water samples. Labelled bands illustrate some instances where cloned and sequenced bands cut from the same position on the gel had the same sequence (same sample-type origins: 3P(2)/3C(1), 3P(3)/3C(2)/3B(2) and 2P(2)/2D(1)) or had sequences belonging to different phyla (different sample type origins: 2 A(1)/3P(1), 2 A(2)/3B(1) and 2P(1)/3C(3)).

band 2/3B band 1 and 2P band 1/3C band 3). Seven of the eight band pairs from within a sample type were not only the same phyla, but were ≥98% similar in sequence identity (examples can be seen in Fig. 2; 3P band 2/3C band 1, 3P band 3/3C band 2/3B band 2, and 2P band 2/2D band 1).

Clone libraries of 16S rRNA genes revealed distinct assemblages for each sample type. The greatest number of phyla per library were observed among clones from the coral samples (range: 6–8; mean = 7) while fish feces contained the lowest number (3; Fig. 4). More than 60 taxonomic families were observed among 568 total 16S rRNA gene sequences (data not presented). Most families were confined to a single sample type from a single site, but some were represented in multiple samples from multiple sites.

Proteobacteria composed the greatest portion of phyla among clones from the coral samples, the free-living fraction water samples, and the feces sample (Fig. 4). Sequences that comprised the proteobacteria portions were different depending on sample type. Most proteobacteria sequences from milkfish feces were *Vibrio*, while sequences from the three free-living water samples and the four coral samples contained multiple proteobacteria taxa with few *Vibrio (*data not shown). *Cyanobacteria* sequences dominated all three particle-attached fraction water samples, and each of the particle-attached water libraries was significantly more similar to each other than to any of the free-living water libraries (RDP-II Library Compare, 80% confidence threshold; Cole *et al.*, 2007)

All 16S rRNA gene sequences from each clone library were compared by BLAST to the NCBI nt database to determine the percentage of 'novel' bacterial sequences (defined as less than 93% similar to any other sequence

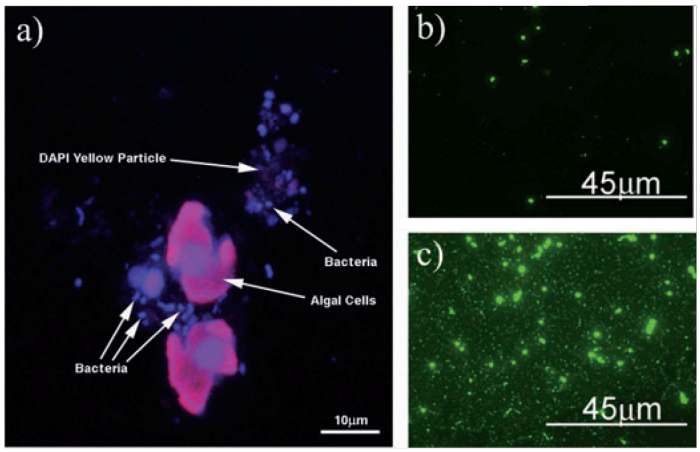

Fig. 3. Scanning laser confocal microscopy allows three-dimensional images to be taken of particles with attached bacteria. A representative maximum-projection confocal image of a DAPI-stained particle from the fish pen site (A) illustrates how bacteria can be enumerated on all sides of the particle. Standard epifluorescence microscopy using SyberGreen (Molecular Probes) stain on free-living microbial assemblages (0.02 µm pore size) reveals a sevenfold difference in the abundance of viruses between the site farthest from the fish pens (Site A; B) and the coral reef site closest to the fish pens (Site D; C).

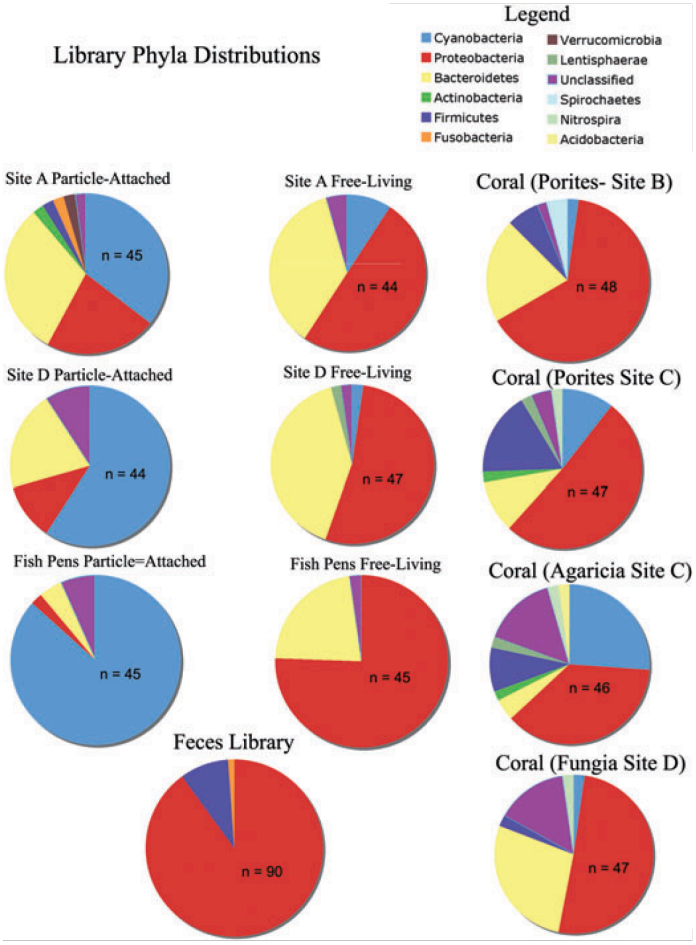

Fig. 4. The distribution of phyla in each 16S rRNA library (from particle-attached water, free-living water, coral tissue, and milkfish feces samples) where the number of sequences analysed for a single clone library is noted in the pie chart.

in the database) for each sample. The proportion of novel sequences ranged from 0% for the Site D water sample to 33% for the AgariciaC31 coral sample. When pooled by sample type, the proportion of 'novel' sequences was 3% for fish feces, 29% for all coral sequences, and 5% for all seawater sequences (Fig. 5). Sequences with high similarity to plastids accounted for large portions of the particle-attached fraction water sample sequences that were not novel (i.e. > 93% similar).

When compared by BLAST with the NCBI nt database, some of the 16S rRNA sequences had high similarity matches (> 97%) to sequences that were previously observed in coral reef ecosystems. Among the four coral-derived 16S rRNA clone libraries, high similarity matches were found to 18 existing accession entries (Table 3). Water and feces libraries each contained a high similarity match to one accession entry (Table 3). In some cases, multiple sequences matched the same accession entry. In total, 20 out of 184 (~11%) sequences matched with high similarity to sequences derived from coral reefs. For water and feces clone libraries, the result was two out of 288 sequences (< 0.1%) and three out of 96 sequences (< 0.1%) respectively. Nearly all of these high similarity matches were to sequences from uncultured microorganisms.

Sequences from 40 cultured bacteria isolated from the water column along the gradient were divided evenly between the phyla *Bacteroidetes* and *Proteobacteria*. There was no apparent pattern in the distribution of these phyla across sites (data not shown). Isolated genera included *Vibrio* sp., *Alteromonas* sp. and *Erythrobacter* sp. The 16S sequences for two of the isolates were highly similar to sequences from other samples (see below; Table 4).

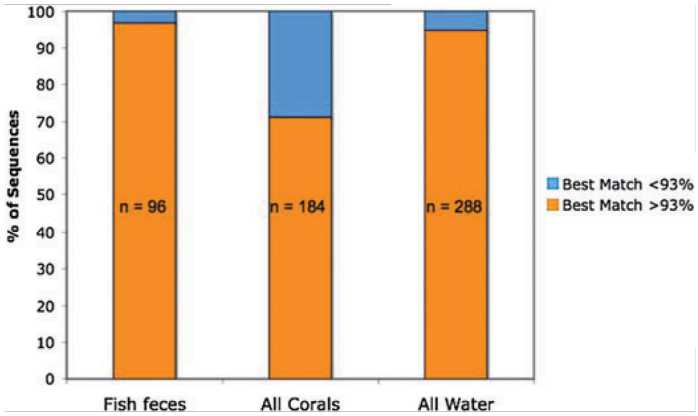

Fig. 5. Percent of novel sequences in 16S rRNA clone libraries. 'Novel' is defined as those that had < 93% best match similarity to the NCBI nt database. About 30% of sequences from coral samples were novel. The total number of sequences analysed for each sample type is presented.

Highly similar shared phylotypes among different sample types

Highly similar 16S rRNA sequences were identified from different sample types. Among 568 sequences, nine matches at ≥99% sequence similarity (over a minimum of 400 bases; Acinas *et al*., 2005) were found in multiple libraries of different origin (i.e. coral, feces or water bacterial communities) (Table 4). Four of the matches were *Proteobacteria* sequences. An uncultured *alphaproteobacterium* (98% similar to Accession No. AM259743) was present in the free-living water fraction collected at Site A and in the *P. cylindrica* collected at Site B. A *Vibrio ponticus*-like bacterium (98% similar to Accession No. AJ630203) was isolated from the water at Site A and also observed in the fish feces library. A sequence 99% similar to *Acinetobacter johnsonii* (Accession No. AB099655) was observed in the milkfish feces library and the libraries of corals collected at Sites B (*P. cylindrica*) and C (*Agaricia* sp.). One *Ralstonia* sp. sequence (99% similar to Accession No. DQ232889) was found multiple times in the feces library and the *P. cylindrica* library from Site B.

Three *Bacteroidetes* sequences were overlapping: one uncultured sequence (98% similar to Accession No. AM238598) was in three samples collected at Site D (free-living water, particle-attached water, and a *Fungia* sp. coral); an uncultured *Flavobacteriales* (99% similar to Accession No. AB294989) was present multiple times in free-living water samples from Sites A, D, and the fish pens as well as in a *Porites cylindrica* collected at Site B; and a *Formosa* sp. sequence (99% similar to Accession No. AY576730) was isolated from water at Site B and also found in the libraries of particle-attached and free-living water samples taken at Site A.

In addition to *Proteobacteria* and *Bacteroidetes* sequences, an uncultured *Firmicutes* (99% similar to Accession No. EF016847) was found multiple times in the fish feces library and a *P. cylindrica* collected at Site B as well as in the *Fungia* sp. library collected from Site D. Also, an uncultured marine bacterium (99% similar to Accession No. EU010165) was found in the free-living water sample taken from Site A and the *P. cylindrica* from Site B.

Discussion

We observed dramatic differences in water characteristics across the transect sites that strongly correlated with distance from the fish pens. The incremental nature of these differences suggests there is a gradient of fish pen influence across the five study sites. Dissolved organic carbon concentration was twofold less at site A relative to the fish pens located 10 km away, while TN at site A was fourfold less than at the fish pens (Table 1). Furthermore, POC concentrations at site A were threefold less than at the fish pens, and the composition of the POC at site A was more detrital material than living carbon as evidenced by the chlorophyll *a* to phaeophytin ratios (Table 1). The chlorophyll *a* concentrations increased more than 40-fold moving from site A toward the fish pens. Virus-like particles increased sevenfold moving toward the fish pens, and free-living bacteria abundances were higher ($P = 0.0006$) at the pens than any other site (Table 2). However, unlike all of the other parameters measured, free-living bacteria abundances were lowest at site C rather than site A ($P = 0.0033$). This non-linear relationship could be due to trophic cascades, terrestrial influence, hydrology, or a combination of any of these factors.

High concentrations of chlorophyll and algal particles at the fish pens suggest a phytoplankton bloom was associated with the fish farms at the time of sampling. Microscopy of the particulate water fraction from site D and the

Table 3. List of 16S rRNA clone sequences with high similarity (> 97%) to sequences previously reported from shallow-water coral samples.

Source	16S clone ID	Closest accession match	Description for 16S rRNA partial sequence	Isolation source
Water	3uD-cl09	EF206913	Uncultured organism clone	Bleached coral (*Oculina patagonica*)(Mediterranean Sea)[c]
Feces	Feces2-clA11	AJ842343[a]	*Enterovibrio coralii* strain LMG 22228T	Bleached coral (*Merulina ampliata*) (Great Barrier Reef)[k]
Coral	**PoritesC32-clC05**[b]	AF365528	Uncultured organism clone	Coral (Bermuda)[d]
Coral	AgariciaC31-clD09	AF365566	Uncultured organism clone	Coral (Panama)[d]
Coral	FungiaD34-clC12	AF365783[a]	Uncultured organism clone	Coral (Bermuda)[d]
Coral	FungiaD34-clH11	AY038410	Uncultured epsilon proteobacterium clone	Water/live coral/dead coral[e]
Coral	AgariciaC31-clE11	AY654762	Mucus bacterium 107	Mucus (*Oculina patagonica*)[c]
Coral	PoritesC32-clC05	DQ117388	Uncultured organism clone	Mucus (*Fungia granulosa*)(Red Sea)[f]
Coral	FungiaD34-clF10	DQ416434	Uncultured organism clone	Coral (*Oculina patagonica*)(Mediterranean Sea)[g]
Coral	**PoritesB32-clH03**	DQ416532	Uncultured organism clone	Coral (*Oculina patagonica*)(Mediterranean Sea)[g]
Coral	**AgariciaC31-clE11**[b]	DQ416571	Bacterium S1cc9	Coral (*Oculina patagonica*)(Mediterranean Sea)[g]
Coral	PoritesC32-clE05	DQ446103	Uncultured alphaproteobacterium clone	Black band diseased coral tissues (*Siderastrea siderea*) (Bahamas)[h]
Coral	**PoritesC32-clD03**[b]	DQ446157	Uncultured alphaproteobacterium clone	Black band diseased coral tissues (*Siderastrea siderea*) (Bahamas)[h]
Coral	PoritesC32-clD03	DQ446158[a]	Uncultured alphaproteobacterium clone	Black band diseased coral tissues (*Siderastrea siderea*) (Bahamas)[h]
Coral	**PoritesC32-clD03**	DQ446160[a]	Uncultured alphaproteobacterium clone	Black band diseased coral tissues (*Siderastrea siderea*) (Bahamas)[h]
Coral	PoritesC32-clE05[b]	EF089420	Uncultured organism clone	Black band diseased coral tissues (*Favites* sp.) (Red Sea)[i]
Coral	PoritesC32-clE05	EF089463	Uncultured organism clone	Black band diseased coral tissues (*Favites* sp.) (Red Sea)[i]
Coral	PoritesC32-clB06	EF089468	Uncultured organism clone	Black band diseased coral tissues (*Favites* sp.) (Red Sea)[i]
Coral	FungiaD34-clD10	EF657852	Uncultured alphaproteobacterium clone	Octocoral (*Eunicea fusca*) (Florida)[j]
Coral	AgariciaC31-clF07	EF657863	Uncultured deltaproteobacterium clone	Octocoral (*Eunicea fusca*) (Florida)[j]

a. More than one clone sequence matched this accession entry at > 97% similarity.
b. For this clone sequence, more than one of the top three BLAST hits had match similarity > 97%.
Clone IDs in bold are > 99% similar to Closest Accession Match.
References: **c.** Koren, O. and Rosenberg, E., unpublished; **d.** Rohwer *et al.*, 2002; **e.** Frias-Lopez *et al.*, 2002; **f.** Kooperman et al., 2007; **g.** Koren and Rosenberg 2006; **h.** Sekar et al., 2006; **i.** Barneah et al., 2007; **j.** Duque-Alarcon, A.P., Santiago-Vazquez, L.Z., Saleh, N.I., Enticknap, J.J., and Kerr, R.G., unpublished; **k.** Thompson *et al.*, 2005.

Table 4. Highly similar 16S rRNA sequences from different sample sites.

Taxonomic ID	Site A	Site B	Site C	Site D	Fish pens
Uncultured *Alphaproteobacteria* (AM259743)	Free-living water	*Porites cylindrica*			
Vibrio ponticus (AJ630203)	Isolate				Feces
Acinetobacter sp. (AB099655)		*Porites cylindrica*	*Agaricia* sp.		Feces
Ralstonia sp. (DQ232889)		*Porites cylindrica*			Feces
Uncultured *Bacteroidetes* (AM238598)				Free-living water, particle-attached water *Fungia* sp.	
Flavobacterium sp. (AB294989)	Free-living water	*Porites cylindrica*		Free-living water	Free-living water
Formosa sp. (AY576730)	Free-living water, particle-attached water	Isolate			
Uncultured *Firmicutes* (EF016847)		*Porites cylindrica*		*Fungia* sp.	Feces
Uncultured marine bacteria (EU010165)	Free-living water	*Porites cylindrica*			

Nine sequences with ≥ 99% similarity (over 400–680 bases) were identified in multiple libraries of different sample type. Taxonomic ID for each sequence was determined from BLAST to NCBI nr/nt database, and GenBank accession numbers (in parentheses) indicate the highest similarity. For Sites B and C, sequences were only obtained from corals and isolated bacteria. For Sites A and D, sequences were obtained from corals, water (free-living and particle-attached), and isolated bacteria. This was the same for fish pens, which also included sequences from fish faeces.

fish pens, as well as qualitative observations of low water visibility at these sites, is consistent with the presence of a diatom bloom. Increased phytoplankton abundances and chlorophyll *a* concentrations are sometimes associated with fish aquaculture in coastal temperate climates (Sara, 2007). The development of blooms may be influenced by dissolved organic nitrogen and carbon from uneaten fish food as well as fish excretions including urea and ammonia (Hall *et al*., 1990). Similar processes likely occur in tropical waters surrounding milkfish cages at Bolinao, and phytoplankton blooms may be one distinguishing characteristic of milkfish cage effluents.We do not know whether the observed bloom was episodic or persistent. Tides may influence bloom variability, though the prevailing water current in Bolinao moves westward from the cages toward site A (Villanueva *et al*., 2005). If bloom conditions are persistent and they extend into reef sites as was observed, then corals may be chronically exposed to fish cage effluents. In that case, particles suspended within fish cage effluents would interact with coral surfaces, and phytoplankton blooms would indicate when these interactions occur with greatest frequency. These events and interactions are of great interest given that similar bloom scenarios in coastal waters can lead to rapid pathogen proliferation, such as *V. cholerae* (Mourino-Perez *et al*., 2003; Worden *et al*., 2006). Whether pathogens can proliferate rapidly in response to organic matter input or phytoplankton blooms in the coral reef waters of Bolinao remains untested.

Particles were highly abundant near the fish pens and the percentage of total bacteria accounted for by particle-attached ranged from 0.1% at site A to 5.6% at the fish pens (Table 2). This contrasts estimates from previous studies that generally found approximately 10% of total bacteria to be particle-attached (Albright *et al*., 1986; Bidle and Fletcher, 1995; Grossart and Simon, 1998; Brachvogel *et al*., 2001; Simon *et al*., 2002). The difference may be due to technology advancements that allowed particle-attached bacteria to be more accurately quantified. Scanning laser confocal microscopy of fluorescently stained particles, as used in this study, permits imaging of particles in three dimensions such that cells can be accurately counted on all sides as well as inside of each individual particle. However, the expected outcome of three dimensional counts would be higher, rather than lower, estimates from those obtained using other methods. Thus, the observed difference in the percentage of total bacteria attached to particles in this system may be a realistic reflection of this ecosystem.

Profiles from DGGE revealed subtle differences in bacteria community composition along the water gradient. The dominant community structure of the water (within particle-attached or free-living fractions) was generally consistent across the gradient (Fig. 2). However, a few prominent bands emerged only near the fish pens and others were present only at sites farthest from the pens. This suggests that the differences observed in the chemical and physical characteristics of the water influenced some members of the microbial communities. While profiles of water samples were quite similar, an important limitation of DGGE was revealed when individual bands were compared. Bands that migrated to a given position generally had identical 16S sequences if they had originated from the same sample type (e.g. particle-attached water), but had different sequences if the bands came from two or more sample types (e.g. particle-attached water versus free-living water). Typically, those differences in 16S sequence were at the phyla level. This

suggests that the dominant members of the microbial communities differed among sample types such that sequences having the same G+C content but completely different sequence order were observed. DGGE was useful in identifying changes in the dominant members of the bacteria communities within a given sample type, but could not be used to reveal meaningful relationships when comparing across sample types.

The 16S rRNA clone libraries provided a survey of the microbial community composition, though comparisons among communities may be limited by low numbers of sequenced clones from each library. Shifts in the microbial community composition of the water column were observed across the sampling gradient. *Cyanobacteria* dominated the particle-attached bacteria community near the fish pens with only two other identifiable phyla represented (Fig. 4). The particle-attached library from the farthest site (Site A) had more than twice the number of phyla than the fish pens and those present were more evenly distributed. A notable feature common to all three particle-attached libraries was a distinct absence of *Vibrio* sequences. This is inconsistent with a proposed conceptual model which posits that vibrios preferentially attach to particles and are not persistent component of the planktonic niche (Nealson and Venter, 2007). The only sample type in which numerous *Vibrio* sequences were observed was the milkfish feces. Two *Vibrio* sp. were among the 40 cultured isolates from whole seawater. Because the seawater was not filter-fractionated prior to culturing, it cannot be distinguished whether the vibrios were free-living or particle-attached.

The clone libraries from the four coral colonies each contained more phyla than other sample types (Fig. 4) suggesting that microbial communities in the corals were more diverse than those in the water or feces. Though coral colonies were collected from three different sites along the gradient, no spatial pattern emerged with regard to coral-associated bacteria phyla. Increased replication for a given coral species and sample site would be required to address this question. Also, more 16S rRNA sequences from each coral sample may reveal phyla that occur in lower abundances. In addition to diversity at the phyla level, the percentage of novel sequences (< 93% similar to any accession entry in NCBI nt database) was highest in the coral libraries (Fig. 5). Some of the observed diversity may be due to polymerase chain reaction (PCR) artifacts, but the clone libraries were checked for chimeras and 99% similarity groups should constrain most Taq polymerase errors (Acinas *et al*., 2005). Thus, most of the observed diversity should reflect the environmental communities that were sampled.

High abundances of novel sequences are consistent with previous observations that the coral holobiont harbours a wide array of undescribed phylotypes (Rohwer *et al*., 2002, Wegley *et al*., 2007). Though databases continue to accumulate 16S rRNA sequences from coral-associated microbial communities, corals remain under-sampled in comparison to the marine water column from which many cultured bacteria have been described. Knowledge of microbial community composition and abundances in healthy corals would benefit the long-term goal of understanding the dynamics of coral pathogens across geographic ranges and fluctuating time scales. If new microbial phylotypes can develop in enriched coral reef habitats, or if the coral environment selects for novel phylotypes, microenvironment niches may play an important mechanistic role in coral mutualism or pathogen development.

Despite the distinct community level differences among all sample types and across the gradient, some 16S rRNA sequences co-occurred among feces, water and corals (Table 4). This finding raises questions regarding the ecology of coral reefs influenced by fish farm effluent. One hypothesis is that the presence of fish pen effluent creates microenvironments that select for specific phylotypes among the diverse samples. For example, microenvironments on coral surfaces may select for bacteria that benefit the coral holobiont, while microenvironments in fish feces and water particulates may independently select for the same phylotypes. An alternate hypothesis is that specific phylotypes are physically transported from the fish pens, attached to fecal particles and phytodetritus, into the coral reef ecosystem and onto coral surfaces. Though some phylotypes that co-occurred in feces and on corals (e.g. *Ralstonia* sp.; Table 4) were not detected in the water samples, the 'transport hypothesis' may still hold true. The observed pattern could occur if feces-associated phylotypes became diluted in the water during transport followed by rapid growth once an appropriate microenvironment is reached. As another example, *Vibrio ponticus* was isolated from water collected at the furthest site (A) and its sequence found in the milkfish feces library, but was not observed in any of the water clone libraries or sequenced DGGE bands (Table 4). It may be possible that fish pen-related phylotypes become so rare in the water column that they were not detected by the molecular techniques used in this study, yet remain viable for isolation on growth media.

The fact that several sequences observed in the coral 16S rRNA gene sequence libraries were highly similar or identical to sequences found previously on corals from a wide geographic range (Table 3) supports the hypothesis that corals exert some selective pressure on their associated microbial community. Furthermore, a particle-attached water sample and a milkfish feces sample each contained sequences previously observed on bleached corals (Table 3). This suggests that some phylotypes capable of associating with corals are also capable of

associating with other surfaces rich in organic matter, such as milkfish feces. Such patterns may also exist in feces from reef fish that closely associate with corals.

Insights into the mechanistic response of coral-associated microbes to organic matter enrichment in the water column may aid predictions of coral reef resilience and resistance capabilities in the face of future perturbations. Further investigation of the mechanisms by which fish cages and coral reefs exchange microbes will be useful in making better informed decisions regarding fish farm placement and creating better management practices for farms located adjacent to sensitive ecosystems.

Experimental procedures

Study site and sampling locations

The study took place in the Bolinao area of the Pangasinan province of The Philippines (16°N, 119°E). Milkfish (*Chanos chanos*) mariculture has been actively practised in the area since 1995 (Holmer *et al*., 2002). The farms employ net pens measuring roughly $10 \times 10 \times 8$ m with a stocking density of approximately 50 000 fish per pen and a pen density of 10 per hectare (Villanueva *et al*., 2005). Farm density is relatively high in the channel between Luzon and Santiago Islands (Fig. 6). Samples were collected immediately adjacent to the fish pens and at four reef sites of varying distance from the fish pens. All reef sites were characterized by low hard coral cover (1%-25%) and low diversity (L. Raymundo, pers. comm.). There are no major river discharges at or up current (south-east) from the fish pens, thus the majority of organic matter input can be attributed to the fish farms (Villanueva *et al*., 2005). Site A is a shallow reef flat located on Malilnep reef ~9 km away from the fish pens. Site B is ~7 km from the pens, and both Sites A and B are sheltered from fish farm effluent by a very shallow sand bar that stretches from Lucero Reef to the north of Silaki Island. Additionally, the prevailing current moves fish farm effluent west-north-west on both ebb and flood tides. Site C is located ~3 km from the pens and on the fish pen side of the sand bar. Site D is ~0.5 km from the pens and directly in the flow of pen effluent.

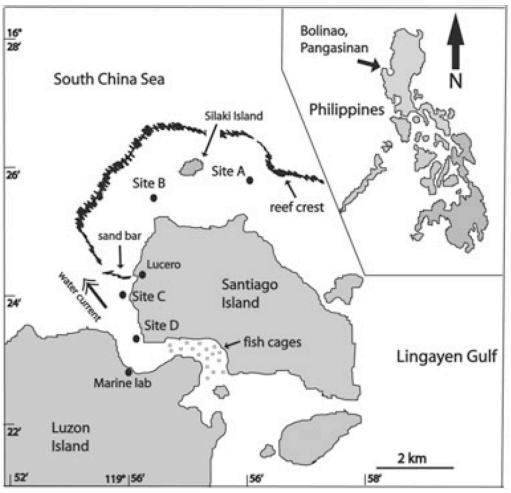

Fig. 6. Location of the study sites in Bolinao, Pangasinan, Republic of the Philippines.

Sample collection

Three 5 L water samples were collected at 1 m depth at each site (fish pens and Sites A–D) between 23–26 January 2007. Four coral samples were collected from Site B a *Porites* sp. (sample B32), from Site C an *Agaricia* sp. (C31) and *Porites* sp. (C32), and from Site D a *Fungia* sp. (D34). One fragment from each colony was placed immediately into an individual sterile Whirl-Pak® bag while underwater. Low live coral cover at each site limited collection of replicate samples. Three milkfish (47.3 ± 7.2 g wet weight; 16.5 ± 1.5 cm fork length) were harvested (29 January 2007) from nearby pens and immediately dissected. Colon contents (hence forth called 'fish feces') from the terminal end of the intestines were collected into ethanol-sterilized dishes using sterile spatulas. For each sample, ~25 mg aliquots were distributed for DNA preservation and dry weight. Fish feces mean percent dry weight was 19.0 ± 2.0%.

Sample preservation for DNA

To preserve fractionated water samples for DNA extraction, 200 ml seawater were filtered onto 3 μm polycarbonate filters and the filtrate was collected onto a 0.22 μm pore size filter (25 mm diameter; Supor 200®; Pall Corp.). To preserve coral samples for DNA extraction, coral fragments were removed from sampling bags in the lab and immersed in ~10 ml filter-sterilized seawater. Slurries (i.e. mixture of tissue and mucus) were removed from the skeleton by plunging repeatedly with a syringe. One ml of slurry was filtered onto a 0.22 μm Supor® filter. Each filter (for both water and coral samples) was preserved in a 2 ml screw cap sterile tube containing 500 μl of RNAlater™ (Ambion) and stored at –20°C. Aliquots of fish feces were also preserved in RNAlater™.

In addition to preserved environmental samples, water samples (100 μl) from each site were spread onto ZoBell media agar and incubated at 30°C. Isolated colonies were picked and transferred into 5 μl Lyse-N-Go™ (Pierce, USA) and aliquots were stored at room temp for 3 weeks before transfer into –20°C for storage.

Chlorophyll

Chlorophyll *a* concentrations were used as a proxy for phytoplankton biomass. Seawater samples (50 ml) were filtered onto Whatman GF/F filters (25 mm) and stored at –20°C until processing. Samples were processed using the method described by Holm-Hansen and colleagues (1965). Briefly,

filters were extracted in 5 ml methanol for 1 h and fluorescence measured using a Turner Designs 700 fluometer. Extracts were acidified and re-measured to determine total phaeophytin.

Dissolved and particulate organic carbon and nitrogen

Seawater aliquots (30 ml) were filtered onto Whatman GF/F filters (25 mm diameter). The filters were wrapped in aluminium foil and stored at –20°C. They were analysed for elemental C-H-N composition using an automated organic elemental analyser (Dumas combustion method; Marine Science Institute Analytical Laboratory, University of California, Santa Barbara). The filtrates were acidified and analysed for total organic carbon (TOC) and TN content (Scripps Institution of Oceanography, Aluwihare Laboratory). Total organic carbon and TN measurements were performed on a Shimadzu TOC-V instrument fitted with an autosampler and TN. Briefly, the concentration of each sample was calculated from an average of four 100 μl injections using a 5-point potassium hydrogen phthalate or potassium nitrate standard curve and certified reference materials (courtesy of Dennis Hansell, Rosenstiel School of Marine and Atmospheric Science).

Bacteria and particle abundances

Water samples were size fractionated on polycarbonate filters. For particle-attached bacteria, 3 μm was operationally defined as the minimum particle size (Bidle and Fletcher, 1995; Crump *et al*., 1998). Two types of particles were quantified: algal cells and 4′,6-diamidino-2-phenylindole (DAPI) yellow particles (DYP; Mostajir *et al*., 1995). The 'particle' fraction was collected by filtering 10 ml of 2% formaldehyde-fixed sample on a 3 μm filter. The filtrate was then put on a 0.22 μm filter to collect the 'free-living' fraction of the sample. These samples were dried, wrapped in aluminum foil and stored at –20°C. A quarter of each filter was prepared for epifluorescence microscopy using Vectashield mounting medium containing DAPI (Vector Laboratories, USA), while the remainder of the filter was archived. For particle abundances, 20 haphazardly chosen fields of view (with one 510×510 μm grid per field) were counted at 200× magnification. Each field of view was photographed to maintain a record of the particle characteristics. Free-living bacteria were enumerated the same way at 600× magnification. Particle-attached bacteria were quantified using a Nikon, Eclipse TE-2000 U scanning laser confocal microscope. Confocal images were analysed using the NIS Elements software program for bacteria abundance and particle size.

Fish feces and coral slurry samples were also observed using epifluorescence microscopy to estimate cell abundances. Formaldehyde-fixed sample dilutions were stained by adding DAPI directly to the solution, then filtered onto 0.2 μm filters, rinsed with filtered PBS and mounted to slides using fresh glycerol/PBS solution. For the four coral slurries and one fish feces sample from which DNA was amplified, mean microbial abundance estimates were 4.0×10^5 ($\pm$ 1.2×10^5) cells per ml for corals and 4.4×10^6 cells per ml for feces. As coral slurries were not collected quantitatively, these estimates cannot be used for reliable cell concentration estimates on the coral surface.

PCR amplification of 16S rRNA gene sequences

DNA was extracted from water filters, coral and fish feces samples using the UltraClean™ Soil Kit (MoBio). Eluted DNA was concentrated via ethanol precipitation.

To amplify community 16S rRNA gene sequences from water samples for DGGE analyses, the variable V3 region was targeted using primer 341f with a GC clamp (5′- CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGGGGGCCTACGGGAGGCAGCAG-3′) and primer 534r (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer *et al*., 1993). Each 50 μl PCR reaction contained 2 U of Taq DNA polymerase (Eppendorf 5 Prime kit), 0.4 μM of each primer and 250 μM dNTP final concentration. A modified touchdown PCR (Don *et al*., 1991) was used to amplify directly from sample DNA. An initial 94°C denaturing step for 5 min was followed by 30 cycles of amplification (3 min denaturation at 94°C; 1 min annealing starting at 65°C for the first cycle and reduced 0.5°C per cycle to 50°; 3 min extension at 72°C), and a final extension of 10 min at 72°C. The PCR products were separated by electrophoresis on a 1.0% agarose gel for confirmation of ~200 bp product.

To amplify from all samples (water, coral and feces) for clone libraries of community 16S rRNA genes, universal primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and the Eubacterial-specific primer 1492R (5′-TACGGYTACCTT GTTACGACTT-3′; Weisburg *et al*. 1991) were used. The PCR conditions were the same as described for DGGE except five additional cycles of amplification (3 min at 94°C; 1 min at 50°C; 3 min at 72°C) were included just before the final extension (i.e. 35 total cycles). The PCR products were concentrated via ethanol precipitation and then processed through a three-cycle regeneration step to reduce the likelihood of heteroduplex formation (Thompson *et al*., 2002). Five microlitres of the 10 μl regenerated PCR product was used as template for clone library construction (see below).

To amplify 16S rRNA genes from bacterial isolates preserved in Lyse-N-Go, thawed aliquots (1 μl) were transferred to a PCR tube and submitted to a thermocycling protocol for lysis as described by Long and Azam (2001; 65°C/30 s, 8°C/30 s, 65°C/90 s, 97°C/180 s, 8°C/60 s, 65°C/180 s, 97°C/60 s, 65°C/60 s, 80°C/hold until the addition of PCR reagents). The lysed aliquots were used as template in a PCR reaction to amplify a ~1460 bp segment of the 16S rRNA gene via a modification of the touchdown PCR protocol (as above). The PCR products were separated by electrophoresis on a 1.0% agarose gel for confirmation of ~1460 bp product. Products were submitted for direct sequencing (University of Hawaii Genomic Services) using primers 515F (5′-GTGCCAGCMG CCGCGGTAA-3′), 530R (5′-GWATTACCGCGGCKGCTG-3′) and 1070R (5′-AGCTGACGACAGCCAT-3′).

Cloning

The PCR products were cloned using Invitrogen's pCR4-TOPO for sequencing kit with Top-10 chemically competent cells following manufacturer's instructions. Colonies were picked and transferred into LB + Kanamycin media containing 10% glycerol for a 12 h incubation at 37°C, then submitted to a commercial sequencing service (Agencourt Genomic

Services, MA, USA). Gene sequencing using either the M13 forward or M13 reverse primers was performed using BigDye Terminator v3.1 (Applied Biosystems), and sequences were delineated using a PRISM™ 3730xl DNA Analyzer (Applied Biosystems). The number of 16S rRNA clones sequenced per library ranged from 48 to 90.

Denaturing gradient gel electrophoresis analyses

The PCR products (~200 bp) were separated by GC-content using a hot-bath DGGE system (CBS Scientific). One hundred nanogram aliquots of PCR products were loaded onto 8.0% polyacrylamide gels in 0.5× TAE buffer (20 mM Tris, 10 mM sodium acetate, 0.5 mM Na_2EDTA, pH 8.2) with top-to-bottom denaturing gradients of 25–65% formamide and urea [100% denaturant being 40% (v/v) formamide and 7 M urea]. Electrophoresis was run at 80 V for 16 h at 60°C in 0.5× TAE. After electrophoresis, the gels were stained for 30 min in 0.5× SYBR Gold (Invitrogen) in TAE buffer. Gels were then imaged using a UVP Epi-chemi Darkroom with a charge coupled device camera. Image exposure times were adjusted depending on light saturation for the most intense band.

Community DGGE profiles were compared across sites and sample type using a standard ladder made from 100 ng each of two isolates collected from the fish pen site. Bands of the same relative position among sites or sample type were identified. These select bands were excised from the gel and eluted using the protocol described by Long and Azam (2001). The bands were re-amplified and run on a new gel to confirm the position relative to a known standard. For those products of the correct position, the original excised band was again amplified and the product used for cloning reactions (see Cloning).

Sequence analyses

Sequences were trimmed, cleaned, and aligned using Sequencher 4.5. Final clean sequences were exported in FASTA format and imported into ARB (Ludwig *et al.*, 2004). ARB was used to align sequences to the Jan04 corrected database and imported into the 14 000 sequence tree via the parsimony method. Where sequences from the various sample types clustered together, those sequences were manually aligned in Sequencher 4.5 and were queried against the NCBI nt database using BLAST to determine putative identification. Clusters of 99% similarity were used to identify sequences from the same organism to eliminate bias induced by Taq polymerase error (Acinas *et al.*, 2005). To determine phyla designation, clone library trace files were imported, aligned and classified using the Ribosome Database Project (RDP-9) online portal (Wang *et al.*, 2007). All sequences used in this study were submitted to GenBank (accession numbers are EU636383–EU636472 for feces library, EU636473–EU636514 for Agaricia Site C library, EU636515–EU636560 for Fungia Site D library, EU636561–EU636608 for Porites Site B library, EU636609–EU636654 for Porites Site C library, EU627846–EU627889 for free-living bacteria library from Site A water, EU627907–EU627953 for free-living bacteria library from site D water, EU627954–EU627998 for free-living bacteria library from fish pen water, EU627999–EU628043 for particle-attached bacteria library from Site A water, EU628044–EU628087 for particle-attached bacteria library from Site D water, EU628088–EU628132 for particle-attached bacteria library from Site A water, and EU627890–EU627906 for all bacteria isolated from the water column).

To determine 'novel' 16S rRNA sequences, trimmed sequences were queried against the NCBI nt database using BLAST. The top three matches for each sequence were retrieved, and sequences whose highest BLAST match was less than 93% similar were considered 'novel.' Putative 'novel' sequences were checked for chimeras using the RDP-II Chimera_Check (Cole *et al.*, 2007).

The NCBI database was also used to identify sequences that had previously been observed in coral reefs. To do this, a list of accession numbers was compiled for sequences whose highest BLAST score was greater than 97%. The accession numbers were submitted to NCBI Entrez using the Batch tool. Within the GenBank entry for each retrieved accession, text searches were done for the terms 'coral' and 'mucus'. This method to determine 'coral reef' sequences may not be comprehensive given the recent availability of various environmental metagenomic databases.

Statistical analyses

The software program SAS was used for statistical analysis of data from water characteristics. A Sharpiro–Wilk test was used to test for normality of the data set and a Levin test was used to check the homogeneity of variance. As a preliminary test of significance, an analysis of variance (ANOVA) was performed to quantify the effect of 'site' on total particle abundance, algal particle abundance, DYP abundance, free-living bacteria abundance, chlorophyll concentration, as well as TN and TOC concentrations in the dissolved organic matter fraction. A *post hoc* least squares means test with a Tukey adjustment was performed on any ANOVA that had a *P*-value less than 0.05 to determine the pairwise effect of site on each dependent variable.

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Chapter 5

Resilience of coral-associated bacterial communities exposed to fish farm effluent

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Resilience of Coral-Associated Bacterial Communities Exposed to Fish Farm Effluent

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Abstract

Background: The coral holobiont includes the coral animal, algal symbionts, and associated microbial community. These microbes help maintain the holobiont homeostasis; thus, sustaining robust mutualistic microbial communities is a fundamental part of long-term coral reef survival. Coastal pollution is one major threat to reefs, and intensive fish farming is a rapidly growing source of this pollution.

Methodology & Principal Findings: We investigated the susceptibility and resilience of the bacterial communities associated with a common reef-building coral, *Porites cylindrica*, to coastal pollution by performing a clonally replicated transplantation experiment in Bolinao, Philippines adjacent to intensive fish farming. Ten fragments from each of four colonies (total of 40 fragments) were followed for 22 days across five sites: a well-flushed reference site (the original fragment source); two sites with low exposure to milkfish (*Chanos chanos*) aquaculture effluent; and two sites with high exposure. Elevated levels of dissolved organic carbon (DOC), chlorophyll *a*, total heterotrophic and autotrophic bacteria abundance, virus like particle (VLP) abundances, and culturable *Vibrio* abundance characterized the high effluent sites. Based on 16S rRNA clone libraries and denaturing gradient gel electrophoresis (DGGE) analysis, we observed rapid, dramatic changes in the coral-associated bacterial communities within five days of high effluent exposure. The community composition on fragments at these high effluent sites shifted towards known human and coral pathogens (i.e. *Arcobacter*, *Fusobacterium*, and *Desulfovibrio*) without the host corals showing signs of disease. The communities shifted back towards their original composition by day 22 without reduction in effluent levels.

Significance: This study reveals fish farms as a likely source of pathogens with the potential to proliferate on corals and an unexpected short-term resilience of coral-associated bacterial communities to eutrophication pressure. These data highlight a need for improved aquaculture practices that can achieve both sustainable industry goals and long-term coral reef survival.

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Introduction

Reef-building corals are just one of many animals that have a mutualistic microbial community. However, the particular relationship between corals and their associated microbes may have a more direct linkage to ecosystem health compared with other species' symbiotic microbial community. The ecosystem-level influence of coral-associated microbial communities is rooted in the fundamental role that scleractinian (stony, reef-building) corals play in physically structuring the habitat and supporting reef organisms, and the roles coral-associated microbes have in maintaining holobiont health. In recent years, molecular methods have greatly expanded our ability to study coral-associated microbial communities. We now know some coral species-specific associations of microbes exist [1], that coral-associated communities are distinct from water column-associated microbes [1–3], and that the bacterial communities of corals can shift under conditions of stress or disease [4–7]. The causative agents of many coral diseases remain unknown [8,9], as do the outcomes of interactions among coral-associated microbial communities and various environmental perturbations, such as nutrient enrichment.

Coral disease [10] and nutrient enrichment of coastal waters [11] actively contribute to the continued decline of coral reefs globally. As coastal development and coral disease incidence continue to rise [12], understanding the mechanisms linking them [13] and managing reefs for long-term survival become pressing management priorities.

Managing for resilience is a current priority in the field of coral reef management [14,15]. The goal is to develop and employ management strategies that increase the ability of reef ecosystems

to withstand and recover from stress. Three cornerstones have been proposed to aid the empirical assessment of 'resilience': biodiversity, spatial heterogeneity, and connectivity [15]. The focus has been on broad reef-wide or region-wide assessments [16], but a potentially important and overlooked component to consider is the resilience capability of the individual corals that comprise the reefs. At this scale, we must consider coral-associated microbial communities [4,17]. The same three cornerstones of resilience can also be applied to the microbial communities within a single coral colony. Here we define resilience as the ability of the microbial communities to return to their original composition after disturbance by nutrient enriched waters despite the continued presence of this enrichment. The large body of work illuminating the role of coral-algal symbioses in large-scale bleaching patterns [18,19] is a good example of how understanding the biodiversity, spatial heterogeneity, and connectivity of single-celled dinoflagellates (zooxanthellae) provided critical links between the small-scale processes of coral-algal associations and region-wide bleaching patterns. The discovery of a vast genetic diversity of zooxanthellae led to the realization that not all algal symbionts of corals are equally tolerant to environmental stressors. The distribution of algal symbionts that were more susceptible to increased temperature stress explained not only the single colony-scale bleaching patterns observed in some corals [18], but also helped explain larger region-wide bleaching patterns. It has become clear that the zooxanthellae diversity and distribution patterns of a reef are worth considering when planning management strategies for a given region [20]. The parallel body of work for coral-associated bacteria, archaea, and viruses is much younger and smaller than that of zooxanthellae. We currently understand more about the biodiversity of the coral-associated microbes than we do about the spatial heterogeneity or connectivity of these communities [21]. Thinking about the microbial ecology of these microscale ecosystems in the context of the three resilience criteria will help illuminate the functional role these organisms play in coral health and disease.

This study builds on previous research that demonstrated that coral reef water-associated microbial communities were influenced by effluent from coastal milkfish (*Chanos chanos*) aquaculture in Bolinao, Philippines [17]. Since the corals that lived in the channel before the intensive aquaculture industry began have died out (E. Gomez, pers. comm.), we simulated the effect of the introduction of these fish pens on corals by transplanting live, clonally replicated corals from a relatively effluent-naïve site (Reference site) to sites routinely exposed to some amount of effluent (low exposure as sites Far-1 & 2; high exposure at sites Near-1 & 2). We investigated the biodiversity of coral-associated bacteria communities over 22 days in response to this chronic effluent exposure. This allowed us to investigate both the effect of spatial proximity to fish pens and temporal transitions in microbial communities. We tested the hypotheses that: 1) exposure to fish pen effluent would cause shifts in coral-associated bacterial community composition towards phylotypes not normally associated with healthy corals; 2) corals exposed to high levels of effluent would be colonized by fish pen-associated bacteria; and 3) there would be some degree of resilience in bacterial communities allowing corals to maintain or recover their original microbial communities despite the stress of effluent exposure. This study broadens our knowledge of the links among environmental conditions, bacterial community composition, and coral health. In the face of rising coastal development and changing environmental stimuli, understanding these connections will aid in developing effective and ecologically sound coral reef management strategies. A focus on microbial community shifts in response to organically enriched effluent will contribute to understanding the connectivity of these communities on coral reefs, further advancing our understanding of how to manage for increased resilience.

Results

Fish Pen Effluent is Persistent, Spatially Extensive and Traceable in Real Time

Weekly mapping at 20 stations all around Santiago Island showed that the strongest chlorophyll *a* signals consistently occurred in the channel containing the majority of fish pens (Fig. 1). There are no major river discharges or other point-sources of nutrient enrichment at or up-current (south east) from the fish pens [22], thus the majority of organic matter input can be attributed to the fish pens. An elongated plume of chlorophyll *a* in the direction of the prevailing current was also consistent with the hypothesis that the fish pens were the source of enrichment in this system.

Bacterial abundance, frequency of dividing cells (FDC), cyanobacteria abundance, virus-like particle (VLP) abundance, concentrations of colony forming units (CFU) of *Vibrio* and kanamycin-resistant bacteria, dissolved organic carbon (DOC) concentration, and chlorophyll *a* concentrations at sites along a strong fish pen gradient were all higher during sampling in May and June 2008 than a previous sampling period in January 2007 ([17],Table 1). A pronounced wet season (May and June 2008) influenced the gradient of fish pen effluent distribution. These data also suggested that corals at the most well-flushed sites (such as the Reference site; Fig. 1) in our study area were no longer living in an oligotrophic system. The DOC, bacteria, VLP, and chlorophyll *a* concentrations were all indicative of a mesotrophic or eutrophic ecosystem. The relatively high percentage of total cells that were dividing at any given time point at any site (ranging from $8.6\pm3.1\%$ at Far-2 site to $17.0\pm1.1\%$ at the Fish Pens) indicated that the enriched DOC observed at all sites (ranging from 77.5 ± 0.8 μM at Far-1 to 189.8 ± 1.0 μM at Fish Pens) was readily utilizable to the microbial community to support growth. A truly oligotrophic environment normally exhibits DOC concentration of ~40 uM [23], which is much lower than our relatively well-flushed sites.

Coral-Associated Bacteria Communities Shift in Response to Effluent

One fragment out of the 40 transplants died at site Near-2 before the 5-day sampling point (T-5 days). All other fragments appeared visually healthy throughout the duration of the experiment. Large community shifts in the coral-associated bacteria samples were observed via denaturing gradient gel electrophoresis (DGGE) analysis five days after transplantation. Profiles were most radically different at the Near sites. However, changes were observed at all sites including the Reference, indicating a transplantation effect in addition to an effluent effect. By 22 days post-transplantation (T-22 days), all coral-associated microbial community profiles had shifted back towards their original profile patterns observed at T-0 (Fig. 2).

All corals transplanted to the Near sites at T-5 days showed, via DGGE profiles, a prominent band that matched the 16S rRNA sequence of the coral black band disease (BBD)-associated [24] bacterium *Desulfovibrio* sp. ([15]; Accession No. AY750147.1) with 100% similarity and 100% query coverage. The majority of the bands for this phylotype were present in coral fragments transplanted at sites Near-1 and Near-2 at T-5 days. This genus was not detected with genus-specific polymerase chain reaction (PCR) primers in the bacterial communities associated with the

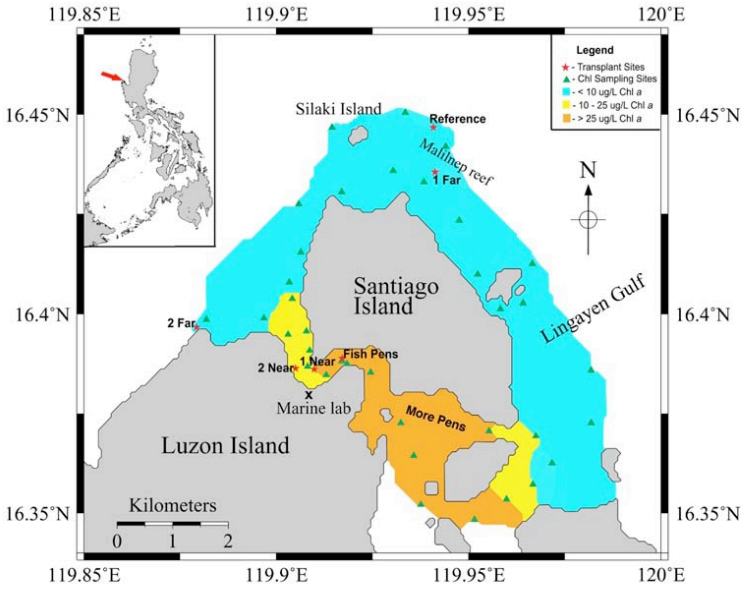

Figure 1. A map of the study sites and surface water *in vivo* chlorophyll *a* measurements averaged over 4 consecutive weekly samplings. Note that transplant site "Fish Pens" did not have corals placed there since no live coral currently exists at that site. It was the location of all water and sediment sampling for the experiment as a representative fish pen.
doi:10.1371/journal.pone.0007319.g001

water column at any site and was only detectable in sediments from sites Near-2 and Fish Pens. Sequences related to the genus were not present in clone libraries for water or sediment bacterial communities. No coral fragment had detectable levels at T-0, but at T-5 days all corals had detectable levels at most sites, identified by genus-specific PCR analysis (Table 2). By T-22 days, only fragments at the Near sites had detectable levels of genus-specific PCR product remaining, the bands in DGGE were greatly diminished or absent, and no sequences were present in clone libraries from that time point.

Evidence suggested that water-associated bacteria from the Fish Pens had the ability to colonize corals exposed to effluent-rich waters. A phylotype most closely related to an uncultured *Roseobacter* (Accession No. EU627982.1) comprised enough of the water-associated bacteria community at the Fish Pens to be detectable via DGGE at T-0, but was completely absent in coral-associated bacteria communities. However, five days after transplantation, this band (verified through sequence analysis) became strongly visible in corals transplanted to Near sites but not in the fragments of the same colony that were transplanted to Far sites (Fig. 3). This same sequence was observed in January 2007 clone libraries from free-living bacteria of fish pen water [17], suggesting that this bacterium may be a persistent feature of fish-pen influenced seawater in Bolinao.

Clone libraries of coral fragments, water, and sediment revealed similar patterns to those seen via DGGE analysis. Very little overlap of phylotypes was observed among water, coral, and sediment bacteria communities. Of the 1172 sequences analyzed from all libraries, there were several noteworthy patterns. Eighteen sequences from the family Vibrionaceae were observed—14 of which came from corals at various site only during the T-5 days time point, while the rest were observed in sediments from the Near sites. Nineteen sequences from the genus *Desulfovibrio* were observed in coral clone libraries– all but two occurrences were from T-5 days and 11 were from fragments at Near sites. Thirty sequences from an order of anaerobes, Clostridiales, were also present among the coral libraries—all but one occurrence were from T-5 days and all but six were from Near sites. Only one Clostridiales sequence was observed in a non-coral library: Far-1 sediment at T-0. Four occurrences of the anaerobic sulfite-oxidizing genus *Sulfitobacter* were observed in a coral at the Reference site ($n = 1$) at T-5 days, and at Near-1 ($n = 3$) at T-22 days. Two genera were coral-specific, appearing in coral libraries from all time points at all sites: *Alcanivorax* ($n = 18$) and *Halomonas* ($n = 334$). Two instances of sequences belonging to genera predominantly comprised of pathogens (human, porcine, bovine, feline, canine, and equine) occurred on corals at the Near sites five days post-transplantation. *Arcobacter*, which is generally associated with feces (human, porcine, and bovine) and has been found in sewage-contaminated waters, was present on a coral at site Near-2 while *Fusobacterium*, often associated with necrotic lesions and a variety of mammal diseases, was present on a coral at site Near-1. Sequences from the class Spirochaete, which have been found previously in diseased coral samples in association with BBD and White Plague-like syndromes [2,4,25], occurred only at T-5 days, mostly at Near sites (Fig. 4).

RDP-10 LibCompare analysis indicated that T-5 days libraries for fragments from different coral colonies at Near sites were more similar to each other than they were to their own original colonies at T-0 or T-22 days ($p \ll 0.05$). In particular, the abundances of Bacteriodetes, Deltaproteobacteria, and Firmicutes were higher at T-5 days than any other time point (Fig. 4). Within a colony, the

Table 1. Comparison of Water Characteristics.

Site	Average values from May and June 2008 (T0, T-5days, T-22days)								Values from January 2007 (Garren et al. 2008)			
	Free-living Bacteria (cells x 10^6/ml±SE)	Virus-like Particles (VLP x 10^6/ml±SE)	Extracted Chl *a* (µg/L±SE)	Dissolved Organic Carbon (µM±SE)	Frequency of Dividing Cells (% total abundance dividing±SE)	Cyanobacteria Abundance (cells x 10^4/ml±SE)	Vibrio (TCBS media) plate counts (CFU/ml±SE)*	Kanamycin resistant plate counts (25 µg/ml; CFU/ml±SE)*	Free-living Bacteria Abundance (cells x 10^6/ml±SE)	Virus-like Particles (VLP x 10^6/ml±SE)	Extracted Chl *a* (µg/L±SE)	Dissolved Organic Carbon (µM±SE)
Ref	1.51±0.03	8.0±0.8	2.8±1.2	93.4±17.2	11.1±2.9	11.3±1.0	60±0	398±43				
Far-1	1.94±0.05	14.2±0.9	3.7±0.1	77.5±0.9	11.9±2.0	7.5±0.8	40±12	1765±1495	0.54±0.03	10±0.7	0.25±0.03	69.7±1.3
Far-2	1.76±0.04	14.8±1.0	4.1±1.6	82.0±4.6	10.5±2.8	9.9±1.2	53±7	285±95				
Near-1	7.43±0.18	81.4±4.3	63.4±12.9	143.6±1.4	12.8±0.8	63.4±4.6	160±23	1008±93	0.61±0.06	70±3	4.5±0.2	141±2.9
Near-2	5.71±0.15	75.9±3.6	60.2±15.3	158.0±2.5	12.4±1.9	52.2±5.9	220±23	1218±238				
Pens	10.25±0.50	112.3±4.7	99.8±25.2	189.8±1.0	16.7±1.4	64.2±8.0	340±46	1413±73	0.99±0.03	61±7	10.3±0.2	162±18.5

Water Characteristics from May/June 2008 compared to January 2007. (* signifies T0 data only).

doi:10.1371/journal.pone.0007319.t001

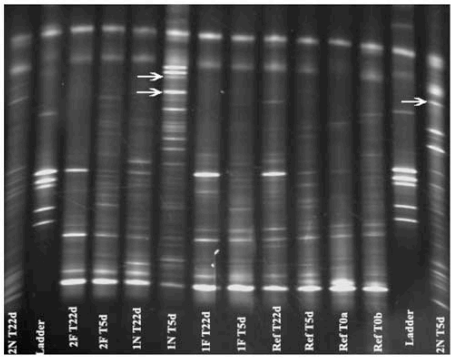

Figure 2. Denaturing gradient gel electrophoresis (DGGE) image of one coral colony (#1) at all sites (Far-1&2, Near-1&2, and Reference) across all time points (T-0, T-5 days, T-22 days). Arrows indicate *Desulfovibrio* bands that were sequence verified. There were two separate fragments sampled from each colony at T0. Both samples are shown on this gel as T0 a and b. Samples from the high effluent sites (Near-1&2) at T-5 days were the only visible *Desulfovibrio* bands.

doi:10.1371/journal.pone.0007319.g002

phylotype distributions were fairly similar among Reference, Far-1 and Far-2 for all time points and Near-1 & 2 for T-0 and T-22 days. The other clone library pattern that revealed itself was an apparent increase in diversity at the high effluent sites at T-5 days. The number of classes represented at the Near sites increased at T-5 days, while the number decreased at the Reference and Far sites (Fig. 4). The Shannon-Wiener index of diversity also increased by 1.7954, from 1.5358 at T-0 to an average of 3.3312 at T-5 days, at the high effluent sites. In contrast, the values for corals at the low effluent sites increased by an average of 0.5140, and by 0.3636 at the reference site.

Discussion

There are several lines of evidence supporting our hypothesis that fish pen effluent induces changes in coral-associated bacterial

Table 2. *Desulfovibrio* Presence/Absence.

Coral & Time / Site	Colony 1		Colony 2		Colony 3		Colony 4	
	T-5d	T-22d	T-5d	T-22d	T-5d	T-22d	T-5d	T-22d
Ref	+	−	−	−	+	−	+	−
Far-1	−	−	+	−	+	−	−	−
Far-2	+	−	+	−	+	−	+	−
Near-1	+	+	+	+	+	+	+	+
Near-2	+	+	+	died	+	+	+	−

The presence (+) or absence (−) of *Desulfovibrio* as detected by genus-specific PCR amplification. No amplified product was detected in any colony at T0, so this time point is omitted from the table for simplicity. The T-22 days fragment for colony 2 died before T-5 days, and was the only mortality for the experiment. *Desulfovibrio* returned to undetectable levels for all colonies at all low-effluent sites by T-22 days (Ref, Far-1&2), yet remained detectable in most colonies at the high-effluent sites (Near-1&2).

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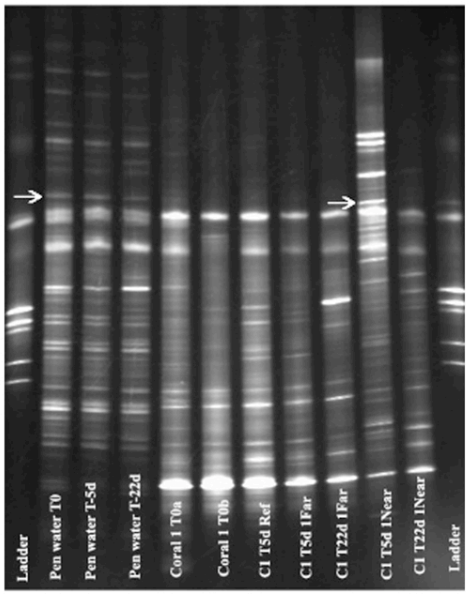

Figure 3. DGGE image showing *Roseobacter* band (>97% sequence similarity) at all time points in the Fish Pen water, but only T-5 days at site Near-1 for coral 1.
doi:10.1371/journal.pone.0007319.g003

community composition. Both DGGE and clone library community composition profiles from all coral colonies at T-5 days at high effluent (Near) sites were more similar to other colonies transplanted to those sites than they were to the communities associated with the fragments from their own colonies transplanted to lower effluent (Far or Reference) sites or sampled from the high effluent sites at the later time point (T-22 days). Corals at high effluent sites were also the only samples with detectable levels of *Desulfovibrio* genus-specific PCR products at the final time point, by which time the same coral clones at lower effluent sites had recovered their original bacterial community composition. This suggests that high effluent exposure was either a source or a facilitator for change in the coral-associated bacterial communities.

The ability of a fish pen-associated *Roseobacter* to become a prominent member of the DGGE profiles from corals at high effluent sites during the first few days post-transplantation was consistent with our hypothesis that corals could become colonized by aquaculture-associated bacteria. Sequences typing pathogenic genera (*Arcobacter* and *Fusobacterium*) generally associated with humans that became associated with the corals were only present at high effluent sites. The fish pens are tended by people who stay in structures directly above the pens. These facilities lack any form of septic or plumbing systems. The presence of potential human pathogens associating with corals suggests that these fish pens represent more than just a source of fish-associated microbes onto the reefs, and that corals are sensitive to these inputs. Beyond the implication for corals, the human health implications of these pathogenic bacteria should not be ignored. These waters are also used for swimming and recreation, thus current aquaculture practices may require improvement for both human and coral health.

A noteworthy observation during this experiment was the restoration of the microbial communities by day 22 towards their

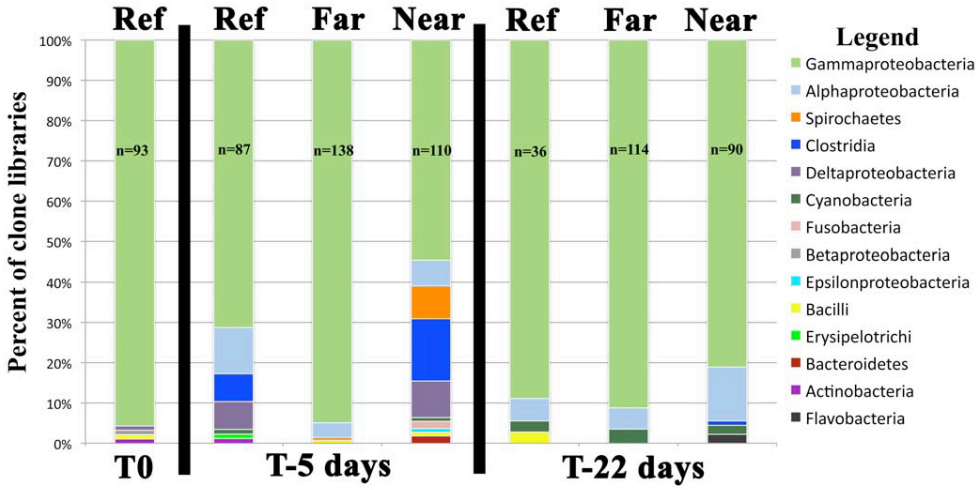

Figure 4. The distribution of phylotypes by class from 16S rRNA clone libraries. Libraries have been pooled by site (i.e. libraries from all fragments at a given time point from Far-1 and Far-2 are represented by "Far" and the same is true for "Near") and each individual time point (T0, T-5 days, T-22 days) is shown separately. The number of sequences represented is denoted as "n = ". Spirochaetes, previously seen only in corals infected with diseases, and Clostridia are both present even though fragments showed no visible signs of disease. Spirochaetes sequences are only present at T-5 days and predominantly at high effluent sites.
doi:10.1371/journal.pone.0007319.g004

original T-0 composition across all sites, despite the surrounding water remaining essentially unchanged. Our original hypothesis was that the low effluent site corals would have the ability to maintain or restore their bacteria community composition, but we observed a much better recovery than anticipated in the high effluent site corals as well. This observation suggests that there is a preferred/stable coral-associated composition that the bacterial community tends toward, and generates hypotheses regarding mechanisms by which a conserved microbial community can be maintained. Whether the coral itself [26–29], its microbial associates [30,31], some combination of the two, or another component or set of components of the holobiont is responsible for restoring the bacterial community balance remains to be understood. However, the observation of the community changing in response to effluent exposure and subsequently recovering *without* effluent reduction suggests that the mechanisms for bacteria community control are strong. Illuminating these mechanisms will be a critical step towards understanding the role of bacterial communities in coral health and disease, and facilitating predictions of reef-wide responses to various stressors.

The fact that the coral-associated bacterial communities at all sites, including the Reference, changed to some degree at T-5 days demonstrated that there was an effect of transplantation. The appearance of *Desulfovibrio* bacteria, generally associated with coral black band disease (BBD), on all coral samples and at all sites at T-5 days was in agreement with our hypothesis that stress, in this case induced by transplantation, would allow corals to be opportunistically colonized by potentially harmful bacteria. Due to the geography and hydrography of the area, the two Near sites had to be located closer to each other than the two Far sites. Thus, one might expect the Near sites to be more similar to each other than the Far sites would be. However, the RDP LibCompare results analyzing clone libraries showed that microbial communities from all fragments at both Near sites at T-22 days were more similar to the communities associated with any fragment from the Far or Reference sites (from any time point) than they were to the communities associated with fragments from the very same colony sampled at T-5 days at that same Near site.

Unlike the *Roseobacter* that was transferred from the fish pen effluent to the coral, the source of *Desulfovibrio* bacteria seems more likely to be the coral itself. Given that *Desulfovibrio* phylotypes were not detectable in water or sediment samples from most sites, yet were detectable in corals at all sites during at least one time point—as it has been detected in corals from around the globe [2,32]— we hypothesize that this potentially detrimental phylotype was originally associated with the corals in undetectable numbers rather than with the surrounding environment. It seems likely that the stress of transplantation, through an unidentified mechanism, allowed these bacteria to proliferate rapidly. However, while transplantation likely initiated the proliferation, exposure to fish pen effluent seems the most likely cause for the continued proliferation of *Desulfovibrio*, as they were visible only at high effluent sites by the final time point. The same mechanisms controlling *Desulfovibrio* proliferation may also be responsible for the proliferation of Clostridia and Spirochaetes in this experiment. The two separate likely sources of newly colonizing bacteria, aquaculture effluent and the coral itself, highlights the potential for multiple pathways of microbial colonization to occur simultaneously. Investigating the ecological interactions among the coral animal, its associated microbiota, and water column-derived microbial invaders may shed light on synergistic roles played by host immunity [26–29,33] and microbe-microbe interactions [9,21,30,31] to protect the holobiont from undesired microbial colonization.

Though all but one of the 40 transplanted coral fragments survived the experiment, it is a worthwhile exercise to consider other potential trajectories this experiment could have taken and the possibilities for these fragments after the final time point. It seems that many of them, especially at the Near sites, may have been particularly vulnerable 5 days after transplantation given the relative increase of sequences affiliated with potential pathogens in molecular profiling results. A previous study showed decreased survival of juvenile corals at this site, and it is possible our corals could have followed a similar fate [22]. Had there been any additional stressor (such as increased water temperature or a major storm event), it is possible that we would have observed a drastically different survival rate. Also, the short time scale of this experiment cannot be extrapolated to imply that these fragments would have survived a long-term transplantation to these sites. The return of the bacteria communities towards their original structure seems to be a positive indication of more resilient capabilities than previously observed; however, the fact that some of the 5 day time point bacteria (such as members of *Desulfovibrio* and Clostridiales) were still detectable at the end of the experiment in corals at high effluent sites should not be overlooked. It is certainly possible that the persistence of these phylotypes could be an early indicator of future disease [34]. Also, the potential physiological costs –such as reduced growth, unsuccessful reproduction, or impaired ability to heal from wounds— of returning the bacteria communities to their original state after disturbance should be considered. There may be hidden costs of this response that we were unable to measure in this experiment.

Observing dramatic changes in community composition over the short time scale of 5 days calls attention to the sensitivity and susceptibility of these corals to physical stress and organic matter enrichment. The lack of visible signs of disease during this stress event raises the need to monitor and observe corals at smaller scales than current monitoring techniques allow. For instance, it may be informative to incorporate microscopy techniques into routine coral reef monitoring protocols. Studying the microbial ecology of sub-lethal and sub-visible effects of stress may provide some of the mechanistic links we need to understand and predict physiological responses of corals to various scenarios. These data provide insight into some of the microbial biodiversity that may be integral in resilience for these corals, and raise the question of whether or not increased diversity of associated bacteria is a desirable state for corals. Our data suggest that an increase in diversity could be indicative of, or correlated to, stress events. These data provide a broader understanding of potentially desirable and undesirable groups of bacteria through time, but they do not yet provide insight into the spatial heterogeneity at the colony scale or connectivity of these microbial consortia. To gain insight into those two resilience criteria, we must shift towards incorporating and developing more sophisticated visual methods of analysis. Some methods that could be incorporated in coral microbiological studies more routinely to this end include fluorescence *in situ* hybridization (FISH), confocal microscopy, and atomic force microscopy. Developing the proper tools to monitor these microscale microbial interactions *in situ* will increase our ability to understand and manage coral reefs in the face of a rapidly changing environment. On a positive note, the ability of the coral-associated bacterial communities to rebound and recover after this severe stress event, without any improvement in the water quality, suggests that corals may be able to survive short, severe stress events if they are not pushed beyond a threshold. The more we learn about individual coral resilience capabilities, the better we can develop management practices integrated across multiple spatial scales for the long-term success of reefs.

Methods

Study Sites

The study took place in the Bolinao, Pangasinan province, Philippines (16N, 119E). Milkfish (*Chanos chanos*) mariculture has been actively practiced in the area since 1995 [35]. The farms employ net pens measuring roughly 10 m×10 m×8 m with a stocking density of approximately 50,000 fish per pen and a pen density of 10 per hectare [22].

A site within the channel between Luzon and Santiago Island was chosen as a representative fish pen sampling site (referred to as "Fish Pens") for water and sediment collection. Two reef sites <1 km from the Fish Pens were selected as sites Near-1 and Near-2 (high effluent exposure) and two reef sites >5 km from the Fish Pens were selected as sites Far-1 and Far-2 (low effluent exposure). The Reference site was ~10 km from the Fish Pens on the outside of the Malilnep reef crest that is regularly flushed by the South China Sea (Fig. 1).

Experimental Design

Four colonies of *Porites cylindrica* were selected from the Reference Site. Twelve branches from each colony were gently removed using wire cutters. Two fragments from each colony were immediately flash frozen as the T-0 sampling. Two additional fragments were transported and affixed in place at each of the following transplantation sites: Reference, Far-1, Near-1, Far-2, Near-2 (note: corals were not transplanted directly at Fish Pens because live coral does not currently exist in that location). The fragments were affixed at each site in flexible plastic tubing with their source colony labeled with Dymo tape and zip-tied to plastic mesh tables installed 1 m above the substrate on rebar supports (Fig. S1). Three replicate tables were installed at each site at 2–3 m depth with the fragments from each colony randomized among the three tables. One fragment from each colony was collected from each site 5 days after transplantation and the second fragment was collected 22 days after transplantation. All fragments were monitored at each visit for visible signs of stress or disease. The experiment took place between May 19 and June 10, 2008.

Sample Collection

Four liter water samples were collected directly above the transplant tables at each site during each sampling period. They were kept cool and shaded until they were processed in the lab within 4 hr of collection. Triplicate sediment cores were collected with sterile 10 ml syringes from each site at T-5 days. Coral fragments were collected in sterile WhirlPaks (Nasco, USA), rinsed with sterile seawater, wrapped in aluminum foil, and flash frozen in liquid nitrogen.

Sample preservation for DNA

A particle fraction was operationally defined as 3 μm being the minimum particle size [36,37]. To preserve water samples for DNA extraction, 200 ml seawater were pre-filtered through a 3 μm-pore size filter (47 mm diameter, polycarbonate, Whatman) and the filtrate was put onto a 0.22 μm-pore size filter (47 mm diameter; Supor 200; Pall Corp.). The 0.22 μm filters were stored at −20°C in 250 μl of RNALater™ (Ambion, USA). To preserve coral samples for DNA extraction, frozen coral fragments were thawed on ice, airbrushed in 2 ml sterile seawater to remove tissue and mucus along the length of the fragment except from the portion that was in contact with the mounting tube, and stored at −20°C. Sediment cores were kept cold until they could be flash frozen in liquid nitrogen within 2 hr of collection and stored at −20°C.

Chlorophyll

Chlorophyll *a* concentrations were used as a proxy for phytoplankton biomass. Chlorophyll *a* was measured both *in vivo* and from extracted samples. *In vivo* measurements were made weekly using a hand-held fluorometer (Aquafluor, Turner Designs Inc., USA) for four weeks at 20 stations (Fig. 1) around Santiago Island to track the influence fish pen effluent. This hand-held *in vivo* fluorometer, coupled with a hand-held Global Positioning System (GPS) unit, allowed for real-time data collection regarding the depth and location of these hotspots. Extracted chlorophyll *a* samples were taken from a subset of these sites each week to create a standard curve that allowed the *in vivo* Relative Fluorescence Unit (RFU) readings to be translated into μg Chl *a*/L. Samples for extracted readings were also taken at each transplant sampling time point. For all extracted samples, seawater (50 ml) was filtered onto Whatman GF/F filters (25 mm) and stored in the dark at −20°C until processing. Samples were processed using the method described by Holm-Hansen et al. (1967). Briefly, filters were extracted in 5–10 ml methanol for two hours and fluorescence measured using a Turner Designs 700 fluorometer. Extracts were acidified and remeasured to determine total phaeophytin.

Dissolved organic carbon

Seawater aliquots (30 ml) were filtered through Whatman GF/F filters (25 mm diameter). The filtrates were acidified and analyzed for total organic carbon (TOC) content (Scripps Institution of Oceanography, Aluwihare Laboratory) on a Shimadzu TOC-V instrument fitted with an autosampler. Briefly, the concentration of each sample was calculated from an average of four 100 μL injections using a five-point potassium hydrogen phthalate standard curve and certified reference materials (courtesy of Dennis Hansell, Rosenstiel School of Marine and Atmospheric Science).

Bacteria and VLP abundances

Water samples were fixed with a 2% final concentration of 0.02 μm filter sterilized formaldehyde. The "particle" fraction was removed by pre-filtering through a 3 μm polycarbonate filter. Two to three milliliters of filtrate were put on a 0.22 μm polycarbonate filter to collect the "free-living" fraction of the sample. These samples were dried, wrapped in aluminum foil, and stored at −20°C. A quarter of each 0.22 μm filter was prepared for epifluorescence microscopy using Vectashield mounting medium containing DAPI (Vector Laboratories, USA), while the remainder of the filter was archived. Bacteria were counted in 20 haphazardly chosen fields of view (with one 100 μm×100 μm grid per field) at 1000×magnification on an Olympus IX-50 microscope. Ten haphazardly chosen fields were photographed at 1000x on a Nikon, Eclipse TE-2000U using NIS Elements software program to count the frequency of dividing cells (FDC) following the protocol described by Hagstrom et al. [38].

Plate counts were performed to quantify the number of culturable bacteria. Water samples (50–200 μl) from each site were spread onto Thiosulfate Citrate Bile Sucrose (TCBS) agar to select for *Vibrios* and Zobell Marine agar with 25 μg/ml Kanamycin to select for Kanamycin-resistant bacteria. Plates were incubated overnight at 30°C and colonies were counted 12 hr later.

Virus-like particles (VLPs) were quantified on 0.02 Anodisc filters (Whatman, USA) that had 500–1,000 μl of sample filtered through them. Filters were stained with 10x SYBR Gold (Invitrogen, USA) for 15 min, dried, mounted to slides using VectaShield (Vector Laboratories, USA). A Nikon Eclipse TE-

2000U and NIS Elements software program were used to image and count 20 haphazardly chosen fields per filter.

PCR amplification:

DNA was extracted from water filters, coral, and sediment samples using the UltraClean™ Soil Kit (MoBio).

To amplify community 16S rRNA genes for denaturing gradient gel electrophoresis (DGGE) analyses, the variable V3 region was targeted using primer 341f with a GC clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCG GGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′) and primer 534r (5′-ATTACCGCGGCTGCTGG-3′) [39] following the amplification protocol described by Garren et al. [17]. The PCR products were separated by electrophoresis on a 1.0% agarose gel for confirmation of ~200 bp product and quantified using PicoGreen (Molecular Probes) following manufacturers instructions using a SpectraMax M2 plate reader (Molecular Devices, USA).

To amplify from all samples for clone libraries of community 16S rDNA genes, a nested PCR was performed using the universal primer 27F (5′-AGAGTTTGATCM TGGCTCAG-3′) and the Eubacterial-specific primer 1492R (5′-TACGGYTACCTT GTTACGACTT-3′; [40]) in a 15 cycle amplification using a 55°C annealing temperature. One microliter of the product was used as the template for the second PCR reaction. Primers 341-forward (without GC clamp [39],) and 981-reverse (41) were used under the same conditions as for DGGE. Three microliters of the final PCR product was used as template for ligation and clone library construction (see below).

To assess the prevalence of this genus in samples that may have had abundances too low to detect via DGGE, we designed specific to probe environmental DNA samples. Genus specific primers were designed using NCBI's Primer-BLAST for a 576 base pair region of *Desulfovibrio spp.* to look for these phylotypes previously associated with Black-Band Disease (BBD) in corals. Primers 645F (CAAGCCCCCAACA CCTAGTA) and 1220R (TACCGTGGCAACGATGAATA) were used in the following PCR protocol: an initial 94°C denaturing step for 5 min was followed by 34 cycles of amplification (45 sec denaturation at 94°C; 45 sec at 53.5°; 2 min extension at 72°C), and a final extension of 10 min at 72°C. Primer specificity was verified via cloning of PCR product.

Cloning

PCR products from half of the fragments analyzed via DGGE were cloned using Invitrogen's pCR4-TOPO for sequencing kit with Top-10 chemically competent cells following manufacturer's instructions. 48 or 96 colonies were picked for each cloning reaction and transferred into LB + Kanamycin media containing 10% glycerol for a 12 h incubation at 37°C, then submitted to a commercial sequencing service (Agencourt Genomic Services, MA, USA). Briefly, inserts were amplified using the M13 forward primer, sequencing reactions were performed using BigDye Terminator v3.1 (Applied Biosystems), and sequences were delineated using a PRISM™ 3730xl DNA Analyzer (Applied Biosystems).

Denaturing gradient gel electrophoresis (DGGE) analyses

PCR products (~200 bp) were separated by GC-content using a hot-bath DGGE system (CBS Scientific). One hundred nanograms of each PCR product were loaded onto 8.0% polyacrylamide gels in 0.5x TAE buffer (20 mM Tris, 10 mM sodium acetate, 0.5 mM Na_2EDTA, pH 8.2) with top-to-bottom denaturing gradients of 35–65% formamide and urea (100% denaturant being 40% [v/v] formamide and 7M urea). Electrophoresis was run at 55 V for 18 h at 60°C in 0.5x TAE. After electrophoresis, the gels were stained for 15 min in 0.5x SYBR Gold (Invitrogen) in TAE buffer. Gels were then imaged using a UVP Epi-chemi Darkroom with a charge coupled device (CCD) camera.

Community DGGE profiles were compared across sites and sample type using a standard ladder made from 100 ng each of two isolates collected from the fish pen site in January 2007 [17]. Bands of the same relative position among sites or sample type were identified. These select bands were excised from the gel and eluted using the protocol described by Long and Azam [42]. The bands were re-amplified and run on a new gel to confirm the position relative to a known standard. For those products of the correct position, the original excised band was again amplified (without GC-clamp) and the product used for cloning reactions (see Cloning).

Sequence analyses

Sequence data were trimmed, cleaned, and aligned using Sequencher 4.5. Final clean sequences were exported in FASTA format and imported into the Ribosome Database Project (RDP-10) online portal [43]. RDP was used to align sequences and classify sequences and to compare libraries. Where sequences from the various sample types clustered together, those sequences were hand aligned in Sequencher 4.5 and blasted in NCBI to determine putative identification. Sequences were submitted to GenBank (accession numbers GQ412750 - GQ413933). 99% similarity clusters were used to identify sequences affiliated with a specific organism to eliminate bias induced by Taq polymerase error [44]. Values for the Shannon-Weiner Index of diversity were calculated for clone libraries using FastGroupII program [45].

Supporting Information

Figure S1 Image showing the transplantation table set-up at the Reference site.

Found at: doi:10.1371/journal.pone.0007319.s001 (7.68 MB TIF)

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Author Contributions

Conceived and designed the experiments: MG LR JG CDH FA. Performed the experiments: MG LR JG. Analyzed the data: MG. Contributed reagents/materials/analysis tools: CDH FA. Wrote the paper: MG. Edited the paper: LR CDH FA.

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Chapter 6

Corals shed bacteria as a potential mechanism of resilience to organic matter enrichment

Abstract

Understanding the mechanisms of resilience of coral reefs to anthropogenic stressors is a critical step toward mitigating the current global decline of these ecosystems. Coral-bacteria associations are a fundamental part of coral health and disease, but the direct observations on the role of these interactions in reef resilience remain largely unexplored. Here we use novel technology, high-speed laser scanning confocal microscopy in situ on live coral (Pocillopora damicornis), to test the hypothesis that corals exert control over the abundance of their associated bacterial communities by actively releasing ("shedding") bacteria from their surface, and that they can use this mechanism to counteract the effects of excessive organic matter inputs. This first report of direct observation with high-speed confocal microscopy of living coral and its active associated bacterial community revealed a layer (3.3 - 146.8 μm thick) on the coral surface above and through the epidermis where bacteria were concentrated. The results of two independent experiments showed that abundance of bacteria in this layer was not sensitive to organic matter enrichment (5 mg L^{-1} peptone), and that coral fragments exposed to enrichment released significantly more bacteria from their surfaces than the controls ($p< 0.01$; an average of $35.9 \pm 1.4 \times 10^5$ cells cm^{-2} coral versus $1.3 \pm 0.5 \times 10^5$ cells cm^{-2} coral). They also increased the rate at which they released particles (transparent exopolymers, TEP) colonized with bacteria ($p<0.05$). Our results suggest that shedding bacteria may be an important mechanism by which corals regulate associated bacterial abundance under organic matter stress. Additionally, the novel ability to watch this ecological behavior in real time at the microscale level at which it actually occurs opens an unexplored avenue for dynamical and mechanistic studies of coral-microbe interactions.

Introduction

Coral reef resilience to the suite of anthropogenic stressors they currently face has been an active area of research in recent years. There have been substantial discussions in the literature about

how to define resilience (Nystrom et al 2008), how to manage reefs in ways that encourage it (Hughes et al 2010), and how to synthesize case studies to elucidate the large scale mechanisms that determine whether a stony coral-dominated ecosystem can resist a phase-shift or rebound from one (Norstrom et al 2009). However, very little attention has been given to the small- scale mechanisms relevant to microbial processes that determine the resilience of individual corals to a given stressor. Delineating the mechanisms by which individual colonies resist or recover from environmental stresses may be a critical link for understanding and predicting larger reef and region-wide patterns of resilience.

Recently, researchers have begun to examine the potential for climate-driven impacts on coral-microbe associations and the corresponding influence on the function of coral-dominated reef ecosystems (Meron et al 2011, Mouchka et al 2010). While climate-driven impacts are of great concern to the longevity of all reefs, excessive organic matter input is another acute global threat that is degrading reefs worldwide. Coral-microbe relationships can be sensitive to organic matter inputs (Thurber et al 2009), and surprising resilience at the single colony level has been observed on short time scales (Bourne et al 2008, Garren et al 2009). Elucidating the underlying mechanisms of resilience to organic pollution may clarify how and why large-scale phase shifts occur on enriched reefs (i.e., what triggers the "tipping point"), and could directly inform management actions.

A surprising finding has been that the abundance of bacteria associated with corals exposed to chronic enrichment from a sewage treatment plant did not harbor more bacteria on their surface than corals at reference reefs (Garren and Azam 2010), and the same has been observed for corals exposed to large amounts of fish-pen effluent that had an order-of-magnitude more bacteria than the typical reef water for the area (Garren et al 2009). There were differences in the community composition between reference and treatment sites, but no significant difference in the abundance. We do know that a tipping point can exist in certain diseased states where corals become more heavily colonized than in their healthy state (Luna et al 2007), but the underlying mechanisms are not known.

The goal of this study was to employ novel technology to test the hypothesis that corals are able to exert control over the abundance of their associated bacterial communities by actively releasing ("shedding") bacteria from their surface. We tested this hypothesis in situ on live coral using a high-speed laser scanning confocal microscope to directly observe and quantify the natural shedding rate and the subsequent response to organic enrichment. To the best of our knowledge, this is the first time that the natural assemblage of coral-associated bacteria have been observed in situ on live coral, and their ecological interactions documented in real time.

Experimental Setup

The reef-building coral Pocillopora damicornis was used for this study. Colonies were cultured at 28°C on a 12 h light/12 hour dark cycle in flow-through seawater aquaria at Scripps Institution of Oceanography, La Jolla, CA. Small fragments (1 – 3 mm in length) were clipped from three individual donor colonies and allowed to acclimate for 48 h. For all experiments, fragments were transferred to individual 50 ml closed-system chambers containing 0.2 µm filtered autoclaved seawater (FASW) with a 0.2 µm filtered air supply. The temperature was maintained by placing the chambers in a 28°C water bath, and light cycling remained the same.

Experiment #1: Influence of Organic Enrichment on Shedding *In Situ*

To observe shedding of bacteria from the coral surface in situ, bacteria were directly quantified on and around the living coral surface using a high-speed laser scanning confocal microscope (Nikon A1-R) with a temperature controlled chamber maintained at 28°C. Observation of live coral-microbe interactions using confocal microscopy had not been successful previous for a combination of reasons that include a strong autofluorescent signal from the coral that can overwhelm signal from stains, and the performance abilities of the last generation of confocal microscopes did not offer the speed, detection sensitivity, resolution, and working distance needed to simultaneously image the millimeter scale coral animal and the micrometer scale bacterial associates in real time. A new generation of confocal microscopes just entered the market this year that

alleviates these barriers. The Nikon A1-R has drastically improved sensitivity, resolution, and speed (imaging up to 150 frames second^{-1} on live coral).

Three coral fragments from separate donor colonies were sampled at T_0, while one fragment from each donor colony was exposed to 5 mg L^{-1} peptone (approximately double the concentration of ambient dissolved organic carbon) in FASW for 24 h and parallel fragments from the same donor colonies were maintained in FASW without peptone for 24 hours as controls. For sampling, fragments were removed from the experimental chambers, quickly rinsed in fresh 28°C FASW, and then stained with 1 µg ml^{-1} 4',6-diamidino-2-phenylindole (DAPI) in FASW for 15 minutes in cover glass bottom chamber slides. Ten different locations on each fragment were imaged in three dimensions (approximately 215 µm x 215 µm x 150 µm) using a 60x water-immersion objective. Individual planes of focus were also imaged at video rate to record the release of bacteria through time. Nikon's Elements software program was used for both acquisition and analysis of images. A Student's t-test was used to evaluate the significance of the difference between the mean abundances of bacteria found on and shed from corals with and without exposure to peptone for both experiments.

Experiment #2: Natural Rate of Particle and Bacteria Release through Two Diel Cycles.

To quantify the rates of bacteria and particle release from corals through time, a larger volume was need than the chamber slides could accommodate. Four fragments from the same donor colony were kept in 25 ml of FASW in 50 ml experimental chambers for 72 hours. Ninety percent of the water (22.5 ml) was replaced with fresh FASW at 1 h, 3 h, 6 h, and then every 6 h following, for a total of 48 h. At T_{48}, the FASW was amended with 5 mg L^{-1} peptone to expose the corals to organic enrichment. Ninety percent of the water was again removed and replaced with fresh FASW plus peptone after one hour (T_{49}), 3 hours (T_{51}), 6 hours (T_{54}) and then every 6 hours after that for a total of 24 hours (T_{72}). The controls consisted of two experimental chambers that never had a coral introduced (the "blanks") to control for possible environmental contamination during the course of

the experiment, and a set of parallel controls for each coral where 2.5 ml of the removed water at each time point was inoculated into a sterile experimental chamber with 22.5 ml of FASW (or FASW + peptone for T_{48} through T_{72}) to detect in situ growth of bacteria in the water column. Ten milliliters of removed water from each sample were fixed with a 2% final concentration of 0.2 µm-filtered formaldehyde at each time-point. A portion was filtered onto 0.2 µm white polycarbonate filters (Millipore, USA), stained with 1x Alcian Blue for mucus-derived transparent exopolymers (Alldredge et al 1993) and 2 µg ml^{-1} DAPI for bacteria, and counted on an Olympus IX-51 epifluorescent microscope. TEP abundance was quantified at 100x magnification while total bacterial abundances and the number of bacteria attached to TEP particles were quantified at 1,000x. The number of bacteria counted in each of the controls was subtracted from the number in the corresponding treatment chamber to account for in situ growth of bacteria in the water column during each time period. The dilution culture approach was necessary to keep growth in the water column low enough to detect bacteria being released from the corals. A portion of unfixed water from each of the 10 chambers was sampled at T_{72} for total organic carbon (TOC). Mucus samples were collected from each fragment after a rinse in FASW at T_0 and T_{72} by air exposure for 3 min over a sterile 1.5 ml tube. The mucus was fixed with 2% final concentration 0.2 µm filtered formaldehyde and bacteria were counted following a previously published protocol (Garren and Azam 2010).

Results and Discussion

Experiment #1

This first report of direct observation with high-speed confocal microscopy of living coral and its active associated bacterial community revealed a layer on the coral surface above and through the epidermis where bacteria were concentrated (Fig. 6-1a). The thickness of this bacteria-dense layer ranged from 3.3 µm to 146.8 µm. It was thinnest on the tentacles and thickest on the coenosarc between polyps. Exposure to organic enrichment did not influence the number of bacteria colonizing the coral surface despite ~7-fold higher numbers of bacteria in the water column of the peptone

enriched experimental chambers at T_{24} compared to the control chambers ($p<0.0001$, an average of $35.2 \pm 1.6 \times 10^5$ cells ml^{-1} versus $5.2 \pm 0.4 \times 10^5$ cells ml^{-1} in the control chambers). There were no significant differences in coral-associated bacterial abundance among any of the fragments from T_0 (ranging from 6.8 to 9.7 x 10^5 cells cm^{-2} coral; or 2.6 to 5.1 x 10^8 cells cm^{-3} coral surface layer), T_{24} controls (4.8 to 15.6 x 10^5 cells cm^{-2} coral; or 1.5 to 2.2 x 10^8 cells cm^{-3} coral surface layer), and T_{24} peptone treatment (7.8 to 19.0 x 10^5 cells cm^{-2} coral; or 1.1 to 4.3 x 10^8 cells cm^{-3} coral surface layer). However, fragments exposed to peptone released significantly more bacteria from their surfaces than control fragments in a given instant ($p< 0.01$; an average of $35.9 \pm 1.4 \times 10^5$ cells cm^{-2} coral versus $1.3 \pm 0.5 \times 10^5$ cells cm^{-2} coral; Fig. 6-1b). These findings underscore the remarkable ability of corals to regulate the abundance of bacteria on their surface in the face of organic enrichment and substantial increases in bacteria in the surrounding water. They also highlight the potential importance of shedding bacteria as a mechanism of resilience to such perturbations.

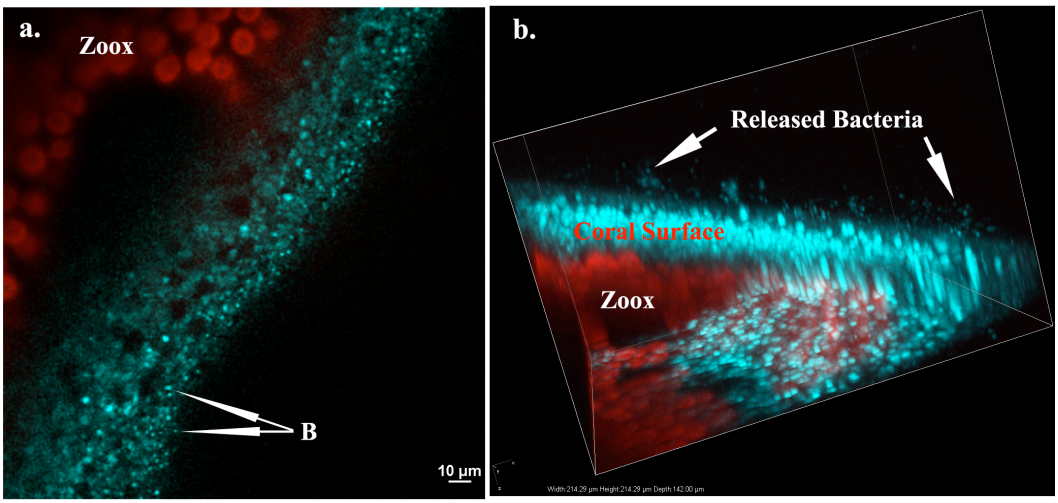

Figure 6-1. Laser scanning confocal images of DAPI stained *P. damicornis*. Arrows point to individual bacterial cells (B) and the symbiotic dinoflagellates (zooxanthellae (Zoox)) appear in red due to the autofluorescence of their chlorophyll pigments. A) This optical slice through the coenosarc of a fragment from the control treatment showing the inner layer of zooxanthellae with an outer crust of abundant DAPI stained bacterial cells on the coral surface. B) This is a three-dimensional rendering of a z-stack from a coral in the peptone treatment after 24 hours of exposure. There are many bacteria, some of which are attached to mucus-like particles, just above the coral surface being released into the water.

Another notable observation was that the frequency of dividing cells (FDC) in each coral-associated bacterial community was extremely high, ranging from 25% (T_{24} controls) to 30% (T_{24} peptone treatment) of the total observed population. Previous studies of coral mucus found the FDC to range from 3 to 7% (Garren and Azam 2010). This observation suggests that the coral-associated bacterial communities may grow extraordinarily rapidly under these experimental conditions. However, we cannot rule out (indeed we consider it likely) that the FDC values were overestimated: we may have missed some bacteria in our effort to obtain reliable counts in the dense mucus layer matrix, and therefore these data underestimate the total abundance and/or preferentially count dividing cells because of their distinctly identifiable cell morphology. In either case, these measurements are minimum estimates of the actual bacterial abundances.

Experiment #2

Over the course of 72 hours, the abundance of bacteria in the surrounding water increased by more than 4 orders of magnitude (from $1.2 \pm 0.6 \times 10^2$ cells ml^{-1} at T_0 to an average of $9.1 \pm 0.6 \times 10^6$ cells ml^{-1} at T_{72}), however the number of bacteria in the coral mucus did not change significantly (an average of $2.2 \pm 0.4 \times 10^5$ cells ml^{-1} mucus at T_0 versus $2.3 \pm 0.6 \times 10^5$ cells ml^{-1} mucus at T_{72}). Shedding bacteria from the coral surface appeared to be an important component in the mechanism by which corals maintain a stable concentration of bacteria on their surface (Fig. 6-2a). The rate of bacteria released increased during the course of the experiment (from an average of $2.8 \pm 0.5 \times 10^4$ cells cm^{-2} of coral h^{-1} during day 1, to $86.7 \pm 12.4 \times 10^4$ cells cm^{-2} of coral hr^{-1} during day 2, to $541.7 \pm 90.0 \times 10^4$ cells cm^{-2} of coral hr^{-1} during day 3 with the peptone enrichment, as did the number of bacteria associated with TEP particles (Fig. 6-2 b-e). The rate of TEP release was not significantly different over the course of the first two days; however, after the addition of peptone, it did increase significantly ($p<0.05$) (from an average of $2.0 \pm 0.5 \times 10^3$ TEP particles cm^{-2} of coral hr^{-1} during day 2 to $6.7 \pm 0.6 \times 10^3$ TEP particles cm^{-2} of coral hr^{-1} during the day with peptone). Because some components of coral mucus stain as TEP (Goldberg 2002), the release of heavily colonized TEP particles suggests that these coral fragments can shed substantial numbers of bacteria as they shed

their mucus. Not all mucus components will stain with Alcian Blue, so there are likely to be many more bacteria attached to mucus particles not observed with this method. Additionally, the coral polyps were filter feeding throughout the experiment and likely removing bacteria and particles from the water column. Given that the corals were the only source of TEP particles in these experimental chambers filled with 0.2 μm filtered sterile seawater, these data provide a minimum estimate of the number of bacteria released on mucus particles.

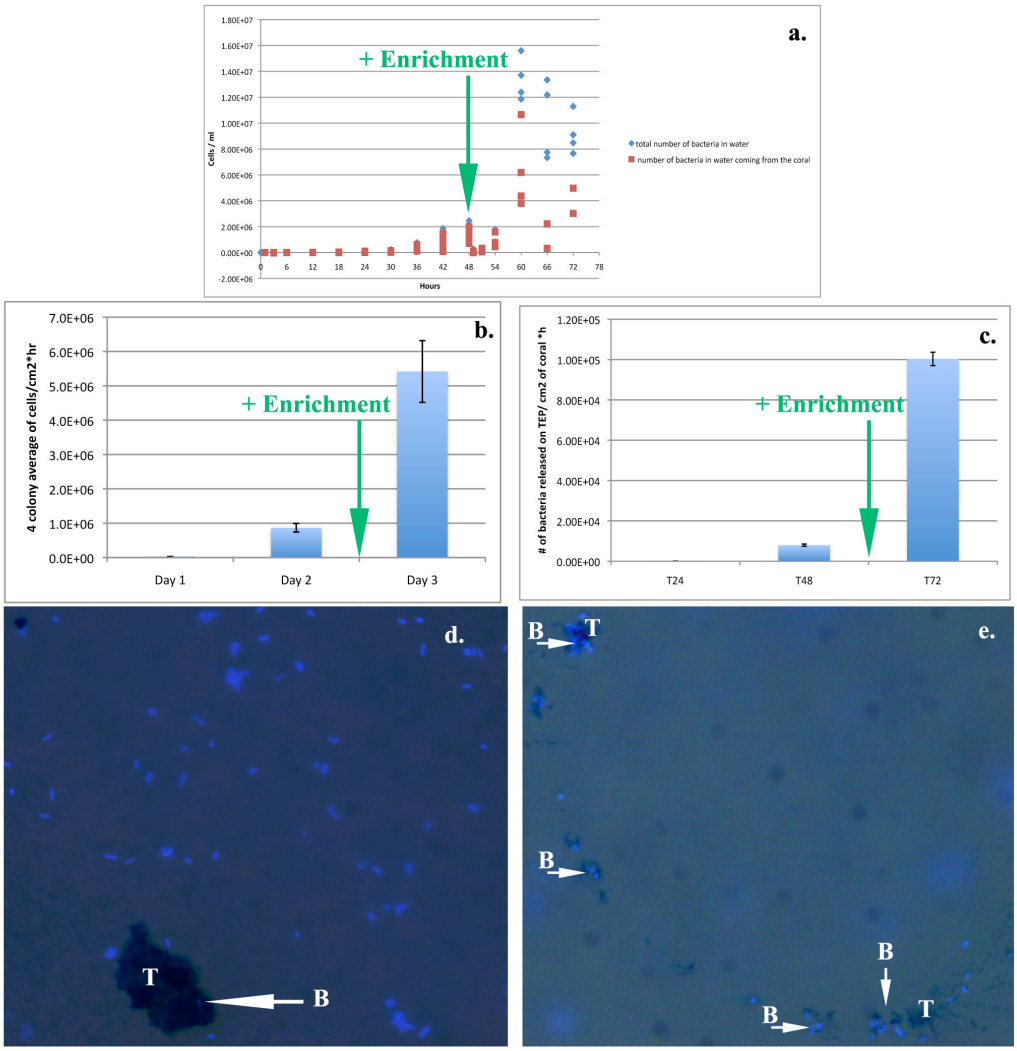

Figure 6-2. These figures are all from Experiment #2. A) The total number of bacteria (DAPI counts) present in the water of the experimental chambers at each time point for each of the four replicates (blue diamonds) as well as the number of those bacteria coming from the coral surface during each time point (red squares). The coral contributes the majority of the bacterial cells to the water until the enrichment addition at T_{48} when in situ growth of bacteria in the water increases. B) The average rate of bacterial cell release per cm^2 of coral per hour for each day. The rate of release increased by ~5-fold after the addition of peptone. C) The average rate of bacteria released on TEP particles per day. The rate at which bacteria were released attached to TEP particles increased by ~12-fold after the addition of peptone. D) This is an overlay at 1,000x magnification of a brightfield image showing Alcian stained TEP particles (T) in dark blue and the same field of view imaged with epifluorescence showing DAPI stained bacteria (B) in bright blue. These are two TEP particles that are not heavily colonized from T_{48}. E) This overlay of brightfield and DAPI images was acquired in the same way as d. and depicts heavily colonized TEP particles (T) from T_{72}.

Conclusions

The evidence from both experiments highlights the remarkable ability of corals to regulate the abundance of bacteria on their surface in the face of organic enrichment stress and drastic increases in water-associated bacterial abundances. Our results suggest that shedding bacteria into the water column is an important component of the mechanism by which corals regulate associated bacterial abundance, and that they have the ability to use that pathway to cope with organic matter perturbations. By increasing the amount of bacteria and mucus (as TEP) released following exposure to organic enrichment, the corals were able to maintain a relatively stable number of bacteria in their surface-associated community. It is possible that this mechanism may also assist corals is warding off pathogens. In addition to identifying one potential mechanism of resilience, the novel ability to watch this ecological behavior in real time at the microscale level at which it actually occurs opens an unexplored avenue for studying coral-microbe interactions.

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Chapter 6 is a short communication in preparation for submission under the title “Corals shed bacteria as a potential mechanism of resilience to organic matter enrichment” with permission from the coauthor, Farooq Azam

Chapter 7

Concluding remarks

The overall picture painted by the previous six chapters is that a previously unrecognized tight regulation is exerted over the abundance of bacteria populations associated with corals, and that this regulation is resilient to some amount of organic matter (OM) stress. Herein, new methods were described for observing bacteria in the coral environment that allowed us to make this discovery and identify one potential mechanism by which the coral animal may exert some control over bacterial abundance in the face of OM enrichment. However, there are likely many mechanisms at play in concert with shedding, and the coral animal is unlikely to be the only structuring force. We know that the community composition of coral-associated bacteria is distinct from the surrounding water community (reviewed in (Ainsworth et al 2010, Mouchka et al 2010)), and indiscriminate shedding of bacteria would not explain how these distinct communities are maintained. There must be other selective mechanisms involved. For example, normal coral-associated phylotypes may have physiological or behavioral responses that allow them a greater probability of remaining attached to the coral relative to water-associated phylotypes during a shedding event. The mechanism by which a shedding response is triggered also remains unclear. Does the coral sense the bacterial density on its surface? Are there external triggers from the environment that induce an increased rate of shedding?

Trophic dynamics are also likely to play an important role. We know that bacteria-bacteria interactions may be important sources of population regulation (Ritchie 2006, Rypien et al 2010), and that there is a large diversity of virus-like particles associated with corals (Davy and Patten 2007, Marhaver et al 2008). However, the dynamics of phage interactions with other microbes on the coral surface is largely unexplored. Eukaryotic protists have also been documented on corals (Harel et al 2008, Kramarsky-Winter et al 2006), but they have received the least attention of all holobiont microbes. It is hard to imagine a bacterial community of the density we find on a coral surface could exist without eukaryotic grazers taking advantage of the proverbial buffet. We will need to place these trophic interactions in the context of other processes regulating coral bacterial populations before a true cause-and-response understanding is attained and predictions can be made about the resilience of such relationships to stress.

At what time scales different mechanisms of resilience are relevant is another question raised by the body of work presented here. All of the experiments presented in this dissertation took place on the scale of hours to weeks, while much of the OM stress experienced by whole reefs happens on the scale of months to years. Understanding the mechanisms of resilience on short time scales lends insight into where the breakdowns may occur when resilience is not observed across longer time scales.

However, it is the long-term stress that appears to do the most damage, not short pulses of perturbation (McClanahan et al 2002, Williams et al). We likely observed some evidence of this at play under the fish pens—healthy coral reef is reported to have existed there in the early 1990s, before the pens (E. Gomez, pers. comm.). The only benthic covering that can now be observed directly under the pens is chest-deep soft black sediment (pers. observation). So although we observed resilience of coral bacterial communities over the course of 22 days of exposure to these pens, concern remains regarding the duration over which such a resilient response can be maintained. It seems likely that the amount of energy it takes for a coral to rebound from such exposure would ultimately be draining over longer timescale and reduce overall fitness. Others have documented such large-scale community-level changes of reefs in response to fish cages (Huang et al 2011). These large-scale changes beg the question "what are the corresponding microscale mechanisms at play in these changes?"

Understanding the interplay of short-term and long-term processes and the corresponding large-scale and microscale responses are as important for our understanding of coral disease as they are for resilience to OM pollution. Many of the same questions we have raised about the mechanisms responsible for healthy coral functioning in the face of OM stress can be asked about the processes responsible for producing a diseased state in a coral. Delineating the fundamental microscale processes that support coral health will help us to understand how they become disrupted in a disease state and are likely to be involved in determining the limits of resilience in other situations.

The ultimate conclusion of this body of work is that the study of coral-microbe interactions is at an exciting and dynamic juncture where new technologies are opening up the possibility of fundamental discoveries. It is finally possible to study these interactions at the microscale, and our big-picture predictive powers will benefit from this. Armed with this knowledge, it seems that microfluidics may be a prime technology for future study of coral-microbe interactions. It has been successfully used to understand the behavior of pelagic marine microbes (Seymour et al 2010), and may be able to shed light on some of the individual bacterial cell behaviors that enable the apparent paradox of massive shedding of bacteria from the coral surface with retention of a specific community. It may also be an appropriate tool for learning how coral pathogens evade mechanisms of resilience to invade the coral surface that other water-associated phylotypes cannot. And thus, the pursuit of understanding and the adventure of science shall continue.

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