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SAN DIEGO STATE UNIVERSITY

Coral reef microbes: the influences of benthic primary producers, nutrient availability, and anthropogenic stressors on community structure and metabolism

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Biology

by

Linda Ellen Wegley Kelly

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2013

The Dissertation of Linda Ellen Wegley Kelly is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

To my boys Brian, Brenny and Aidan: For being the most amazing part of my life

EPIGRAPH

Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning.

-Albert Einstein

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ABSTRACT OF THE DISSERTATION

Coral reef microbes: the influences of benthic primary producers, nutrient availability, and anthropogenic stressors on community structure and metabolism

by

Linda Ellen Wegley Kelly

Doctor of Philosophy in Biology

University of California, San Diego, 2013

San Diego State University, 2013

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Genomic studies of marine microbes have advanced our understanding of community ecology and the vast array of metabolisms microbes utilize for acquiring energy and nutrients in the ocean. The structure of microbial communities overlying coral reefs have been shown to reflect ecosystem health. For example, algal-dominated reefs are inhabited by more pathogen-like microbes. The objective of my PhD thesis was to use metagenomics to investigate the microbial communities associated with the coral animal (Chapter 2) and coral reefs influenced by different nutrient regimes (Chapter 3) and anthropogenic disturbances (Chapter 4).

The Line Island archipelago consists of eleven atolls spanning a latitudinal gradient from 6^{o} north to 11^{o} south. Nutrient concentrations vary across the islands where inorganic nutrient concentrations are approximately five and two-times higher

for nitrogen and phosphorus, respectively on Jarvis (located closest to the equator) compared to Kingman and Flint (located furthest north and south). Bacterial metagenomes were constructed from 26 coral reefs to investigate community differences between reefs on uninhabited versus populated Line Islands and the influence of biogeochemistry on community structure. The distribution of microbial taxa was most strongly predicted by the composition of certain benthic functional groups. Where reefs with higher coral cover observed on Islands Malden, Flint, and Vostok were associated with higher abundances of *Sphingomonadales*. In contrast, Kiritimati reefs which were dominated by turf algae, were associated with higher abundances of *Bacteriodetes*. The microbial community metabolism on LI reefs was shown to be most strongly influenced by geographic distance from the equator. This grouping of community metabolism based on geographic location occurred despite differences in the distribution of taxa present a reef sites. Distance from the equator is strongly correlated with nitrogen and phosphorus concentrations suggesting that nutrient availability is an important driver for community metabolism on Line Island reefs. Metabolic pathways positively correlated with higher nutrients included conjugative transfer, chemotaxis, nitrate and nitrite ammonification, cobalt-zinc-cadmium resistance, multidrug resistance efflux pumps, and ton and tol transport. Low nutrient availability was correlated with metabolic pathways involved in photosynthesis, such as chlorophyll biosynthesis and photosystems I and II. The results from this study suggest that selection of microbial taxa is based on carbon

sources (benthic community composition) and subsequently, specific genes are incorporated for adaptations to nutrient availability in that region.

To better understand how microbial community structure changes in response to environmental perturbations, three reefs that had undergone a coral-algal phase shift in response to ship groundings were investigated. The Line Islands are calcium carbonate coral reef platforms located in an extremely iron-limited region of the central Pacific. Therefore it was hypothesized that iron leaching from the shipwreck debris was enabling the benthic algae to outcompete and overgrow the corals. The reefs surrounding the shipwreck debris were characterized by high benthic cover of turf algae, macroalgae, cyanobacterial mats, and corallimorphs, as well as particulate-laden, cloudy water. These sites also have very low coral and crustose coralline algal (CCA) cover and are call *black reefs* because of the dark colored benthic community and reduced clarity of the overlying water column. A combination of benthic surveys, chemistry, metagenomics, and microcosms were used to investigate if and how shipwrecks initiate and maintain black reefs. Iron concentrations in algae tissue from the Millennium black reef site were 6-times higher than in algae collected from reference sites. Metagenomic sequencing of the Millennium Atoll black reef-associated microbial community was enriched in iron-associated virulence genes and known pathogens. Microcosm experiments showed that corals were killed by black reef rubble via microbial activity. Together these results demonstrate that shipwrecks and their associated iron pose significant threats to coral reefs in iron-limited regions.

CHAPTER 1: Introduction

The beneficial roles of microbes in the coral holobionts. Corals exist as a complex holobiont which includes the coral animal and a suite of microbiota [1]. The most well-established of these symbiotic relationships is that of the coral animal and its zooxanthellae, however bacteria, archaea, protists, endolythic algae and fungi and viruses are also important members of the coral holobiont [1-8]. Corals inhabit oligotrophic of marine waters and these mutualistic associations increase host fitness by providing essential nutrients [9, 10]. Amongst these microbial counterparts, commensal symbionts contribute to inorganic nutrient availability [11, 12] and provide defense from pathogens to the host [13]. For example, coral-associated microbes from all three domains of life were shown to contain the genomic potential to recycle nitrogen [11, 12]. The process of nitrogen fixation by microbial symbionts has also been confirmed on corals by empirical measurements [10, 14, 15]

Conditions that upset the balance of coral associated microbes have detrimental effects on coral health. In a general sense, a shift in microbial community composition is detrimental to corals when the microbes fulfilling beneficial roles in nutrient recycling are replaced by microbes which do not confer those advantages [16]. An investigation which subjected corals to four pertinent stressors demonstrated the replacement of certain groups of bacteria. Here, stressor treatments reduced the abundances *Actinobacteria*, *Cyanobacteria*, and *Firmicutes*, all of which were previously established members of a healthy coral holobiont [17]. More

specifically, if conditions select for microbes of a more pathogenic nature, these microbes may cause opportunistic diseases.

When good microbes go bad: microbially mediated coral mortality. Coral associated microbes are harmful to the host when conditions influence their activity. Experiments in which coral fragments were dosed with elevated levels of labile organic carbon showed that coral mortality was associated with enhanced microbial growth rates on coral surfaces (10-fold increase, [18]). The observation that dissolved organic carbon (DOC) sources caused significantly more coral mortality than inorganic nutrients, such as nitrogen and phosphorus [18, 19] led to more investigations into the greatest producer of DOC on the reef, benthic macroalgae. Corals exude lower concentrations of DOC into the surrounding water column promoting levels of microbial activity suitable to sustain a commensal relationship. In contrast, fleshy macro – and turf algae release greater concentrations of DOC and engender high microbial activity [20]. These increased concentrations of algal-derived DOC provide excess energy to coral associated microbes allowing them to grow unchecked. This hypothesis was supported by experiments conducted by Smith et al. [21] where corals situated adjacent to algae (separated by a 0.02 μm membrane filter), were subjected to increased microbial activity on their surfaces, and subsequently suffered 100% mortality within 48 hours. The hypothesis that coral mortality was microbially mediated was supported by the observation that coral mortality was prevented by the addition of antibiotics. The proposed mechanism causing coral death is hypoxia resulting from microbial respiration, which can be observed on dying coral

fragments [21] as well as at coral-algal interfaces [22]. Thus, the above studies provide evidence that promoting uncontrolled activity of coral-associated microbes is one mechanism whereby algae overgrow corals and subsequently result in coral-algal phase shifts.

Microbial abundances and composition are indicative of coral reef condition. The community structure of planktonic microbes inhabiting the diffusive boundary layer overlying the reef can be indicative of ecosystem health. In 2005, four coral reef atolls representing a gradient of coral condition (i.e., cover and disease prevalence) were investigated. The most pristine reef in this survey, Kingman, was characterized by low coral disease prevalence and by a benthic community where coral and coralline algae dominated. The most degraded reef, Kiritimati, was characterized by increased coral disease prevalence as well as higher abundances of turf and macroalgae. There were 10X more microbes in the water on Kiritimati, than on Kingman. Based on these results, it was hypothesized that coral condition may be related to changes in microbial abundances and taxa in the water column. Metagenomes were sequenced to determine the composition of the microbial community on the Line Island atolls. Taxonomic assignments from the metagenomic data showed a change from a microbial food web similar to the open ocean (*Prochlorococcus* spp. and SAR11-like bacteria) on Kingman to a community dominated by "super-heterotrophs" most closely related to known pathogens like *E. coli*, *Staphylococcus* spp., *Streptococcus* spp., *Enterobacter* spp., and *Vibrio* spp. [23]. This change in the microbial community was associated with the decline in coral cover (~71% hard corals + CCA on Kingman to ~21% on

Kiritimati) and an increase in coral disease prevalence (<5% on Kingman to 7-20% on Kiritimati [24].

My working model posits that reef degradation, and the associated phase-shifts from coral to algal dominance, leads to elevated levels of algae derived DOM at coral-algal interfaces and within the diffusive boundary layer overlying the reef. This condition is multifaceted influencing the competition between corals and algae as well as the composition of planktonic microbes overlying the reef. At the interface, this results in: 1) greater microbial activity, 2) microbially mediated hypoxia which leads to mortality at coral-algal borders, and 3) subsequent overgrowth of algae over compromised / dying coral. In the water column this results in: 1) higher microbial numbers and biomass and 2) a community shift to 'super-heterotrophs', which are potential coral pathogens.

To address this question, I have studied the microbiology on the surface of coral, on different bottom types across a geographic range of 2,500 km, and in response to a local pollutant.

Coral reef microbes are important contributors to the coral holobiont providing essential nutrients and protection. Molecular techniques have been used to demonstrate that corals harbor diverse and in some cases, species-specific microbial communities [1, 3]. However, the functional roles of these microbes are largely unknown. Unlike gene markers (e.g., 16S rRNA gene), metagenomic libraries provide insight into the structure and function of communities [25]. On healthy corals, the gene function of microbes is expected to reflect the ability to degrade carbon sources

found in coral mucus [26-28], remineralization of available inorganic nutrients, as well as genes required for the adaptation to the environment associated with the coral host. In order to ascertain the functional potential in the diseased state, microbial community function needs to be established in healthy corals.

Chapter 2 describes the results from the first metagenomic study to investigate the roles of microbial symbionts associated with a reef building coral. The most exciting result from this study were several gene similarities for nitrogen cycling that were related to members of the coral holobiont from all three domains of life (bacteria, archaea, and endolithic fungi). Corals thrive in extremely nutrient poor regions. This research provided important insight into a mechanism where corals could increase their ability to acquire an essential nutrient by recycling nitrogen through their microbial counterparts.

Coral reef holobiomes: The influences of benthic primary producing macrobes and nutrient availability on microbial community composition and metabolism. Based on the 2008 study investigating the microbial communities on the four northern Line Island reefs [23], I hypothesized that the marine microbial food web on near pristine reefs will be composed of equal abundances of autotrophs and heterotrophs, while degraded reefs will be dominated by 'super-heterotrophs' that are potential coral pathogens. This observation will be reflected at both the taxonomic and metabolic levels. The prevalence of genes encoding virulence genes and hydrolytic enzymes, such as glucosidases, lipases, phosphotases, and proteases will be greater on degraded reefs.

In chapter 3, I tested this part of the hypothesis. Microbial metagenomes were constructed from 26 coral reefs across the Line Islands located in the central Pacific to determine the influences of benthic community composition, as well as biogeochemistry on bacterioplankton community structure. The distribution of microbial taxa was most strongly predicted by the prevalence of certain benthic functional groups whereas; the bacterial community metabolism on LI reefs was shown to be most strongly influenced by distance from the equator. The results from this study suggest that selection of microbial taxa is based on carbon sources (benthic community composition) and subsequently, specific genes are incorporated for adaptations to nutrient availability in that region.

Response of a coral reef community to a local pollutant. Corals and benthic algae are constantly competing for space on the reef [29]. Conditions that enhance the competitive advantage of algae over corals result in coral-algal phase shifts [30]. Competition between corals and fleshy algae (macro- and turf algae) have been shown to inhibit coral growth, decrease fecundity, inhibit coral larval recruitment and survival [31].Though there are conflicting theories as to how algae overgrow live coral tissue, it is well documented that the movement of algae over coral tissues is preceded by a line of tissue damage and necrosis at the interaction zone [22, 32, 33]. The mechanism by which this coral tissue damage occurs has been hypothesized to be the result of physical disruption, allelopathy [34] and microbial activity [20-22].

In chapter 4 the microbial communities associated with three reefs, which had recently undergone coral-algal phase shifts as a result of shipwrecks debris, were

investigated. These reefs are referred to as black reefs because of the turbid water and dark color of the benthos. On black reefs, the microbial communities were characteristic of other degraded reefs (i.e., higher biomass, increased abundances of heterotrophs, and higher concentrations of virulence genes). Similar to the genomic adaptation to nutrient availability demonstrated in the metagenomic study of coral holobiomes from the Line Islands (Chapter 3), there is evidence that the microbes inhabiting the black reef adapted to the increased iron availability. This study supports the growing body of evidence that microbes facilitate overgrowth of live coral by algae. Additionally, I suggest how these results contribute to an explanation of how alternate stable states are maintained on reefs.

Collectively, these three chapters demonstrate the importance of coral- and reef associated microbial communities.

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CHAPTER 2: Metagenomic analysis of the microbial community associated with the coral *Porites astreoides*

The coral holobiont is a dynamic assemblage of the coral animal, zooxanthellae, endolithic algae and fungi, bacteria, archaea and viruses. Zooxanthellae and some bacteria form relatively stable and species-specific associations with corals. Other associations are less specific; coral-associated Archaea differ from those in the water column, but the same archaeal species may be found on different coral species. It has been hypothesized that the coral animal can adapt to differing ecological niches by "switching" its microbial associates. In the case of corals and zooxanthellae, this has been termed adaptive bleaching and it has important implications for carbon cycling within the coral holobiont and ultimately the survival of coral reefs. However, the roles of other components of the coral holobiont are essentially unknown. To better understand these other coral-associates, a fractionation procedure was used to separate the microbes, mitochondria, and viruses from the coral animal cells and zooxanthellae. The resulting metagenomic DNA was sequenced using pyrosequencing. Fungi, bacteria, and phage were the most commonly identified organisms in the metagenome. Three of the four fungal phyla were represented, including a wide diversity of fungal genes involved in carbon and nitrogen metabolism, suggesting that the endolithic community is more important than previously appreciated. In particular, the data suggested that endolithic fungi could be converting nitrate and nitrite to ammonia, which would enable fixed-nitrogen to cycle within the coral holobiont. The most prominent bacterial groups were *Proteobacteria*

(68%), *Firmicutes* (10%), *Cyanobacteria* (7%), and *Actinobacteria* (6%). Functionally, the bacterial community was primarily heterotrophic and included a number of pathways for the degradation of aromatic compounds, the most abundant being the homogentisate pathway. The most abundant phage family was the ssDNA Microphage and most of the eukaryotic viruses were most closely related to those known to infect aquatic organisms. This study provides a metabolic and taxonomic snapshot of microbes associated with the reef-building coral *Porites astreoides* and presents a basis for understanding how coral-microbial interactions structure the holobiont and coral reefs.

Introduction

The coral holobiont [1] is a complex assemblage of the coral animal, microbial eukaryotes such as algae, Fungi, and protozoa, bacteria, archaea, and viruses. The keystone symbiosis for coral reefs is zooxanthellae and coral. By fixing carbon the zooxanthellae provide the animal with energy reserves that are used for constructing the skeleton and producing the mucus sheets [2]. Coral bleaching, one of the major threats to coral reefs, occurs when elevated water temperatures [3], pathogens [4], and other factors [5] cause the zooxanthellae-coral association to breakdown. Global warming is predicted to increase the amount of coral bleaching and poses a direct threat to coral reefs [6]. Buddemeier et al. (1999) have raised the possibility that corals will switch their zooxanthellae for communities more thermally tolerant as environmental conditions change [7, 8]. This Adaptive Bleaching Hypothesis is supported by studies that show ecologically and temporally driven changes in the zooxanthellae associated with corals [9, 10].

Endolithic fungi and algae are microbial Eukarya ubiquitously associated with corals. Presently, it is believed that the coral-associated fungi are parasitic and attack both the coral polyps and endolithic algae [11-13]. However, little is known about the diversity of both endolithic fungi and endolithic algae [14-16] or their contributions to metabolic functions in the coral ecosystem. Protists, including the potential pathogen apicomplexa, are also abundant members of the coral holobiont [17, 18].

The coral holobiont hosts a highly diverse bacterial community [1, 19-22], which in some cases form species specific associations with corals [1, 19, 23]. For

example, a Gamma-proteobacteria designated PA1 was always present in 16S rDNA libraries constructed from *Porites astreoides* [1] and *Porites compressa* (Wegley; unpublished data). Some of this apparent specificity is due to limitations in observing rare species in the environment and some Bacteria have been found on multiple species of corals [24]. Diverse archaeal communities [25-27] are associated with corals. These Archaea are coral-specific in the sense that they do no overlap with water-column Archaea. However, it appears that this association is fairly cosmopolitan with the same Archaea being found on many corals species.

Viruses associated with corals have been shown to be present in coral tissues [28] and have been implicated in coral bleaching and disease [28-31]. Viruses have also been found to be in high abundance on coral surfaces with estimates greater than 10^{10} per square centimeter [32]. Most of these coral-associated viruses are phage, which infect the Bacteria.

While cultured and uncultured based methods have provided details on the diversity and biogeography of coral-associated microbes, the metabolic contributions of these microbes to the function of the coral holobiont are essentially unknown. Rohwer et al. 2002 proposed that the coral holobiont harbors specialized microbiota that may protect the coral animal from pathogens through filling entry niches and/or producing antibiotics and that disrupting this association could lead to coral disease. Corals may also have the ability to switch their prokaryotic communities in order to provide resistance to specific pathogens [33]. Genomic characterization of the coral animal and all its symbiotic microorganisms collectively, has been recommended [34]

for eliciting a better understanding of these complex symbiotic interactions. Metagenomics, or community genomics, provides an opportunity to describe the taxonomic components [35], relative abundances [36, 37] and metabolic potential [38, 39] of all microbes within the coral holobiont. Here we use a metagenomic approach to describe the microbial metagenome of the Caribbean coral *Porites astreoides*.

Results and Discussion

The metagenome of Porites astreoides*:* A mortar and pestle was used to homogenize the *Porites astreoides* fragments and the coral nuclei and zooxanthellae were removed using a combination of filtration and Percoll fractionation (modified from Schirmer et al. [40]). DNA was extracted from the top Percoll layer, which contained most of the microbial-sized cells as determined by microscopy (Figure 1A), amplified using GenomiPhi (Amersham Biosciences, Pittsburgh, PA), and sequenced using 454 Life Sciences pyrosequencing. A total of 316,279 reads, with an average sequence length of 102 base pairs, were obtained from the *Porites astreoides* sample and compared to the SEED non-redundant database using blastx (Table 1) [41]. The majority of sequences (79%; Figure 1B) were classified as unknowns (E-value $<10^{-5}$). The high percent of unknowns is due to the presence of truly unique sequences in the coral holobiont combined with the shorter sequences obtained with pyrosequencing (i.e., the shorter sequence reads reduces the probability of identifying similarities in the databases). The sequences that were similar to sequences in the database (i.e., the

knowns) were grouped as mitochondria (49%), Fungi (38%), Bacteria (7%), phage (3%), eukaryotic viruses (2%), and Archaea (<1%; Figure 1B).

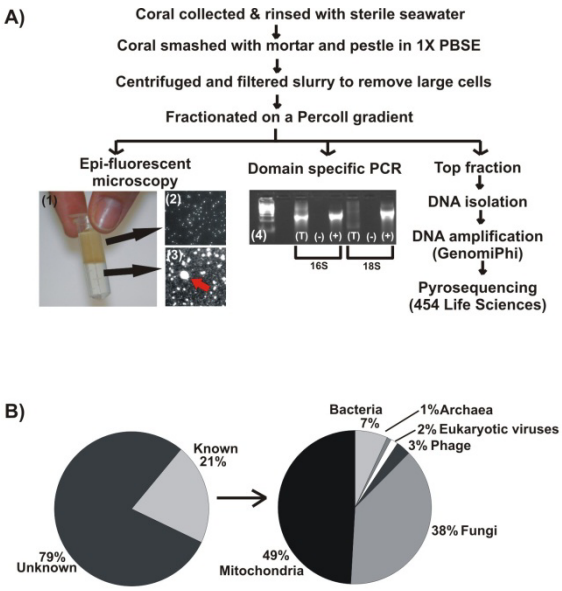

Figure 2.1. Flow chart of the methods used to sequence the microbial metagenome associated with Porites astreoides (A) and the initial blastx results (B). The microbial community was retained in the top Percoll fraction (A1; micrograph A2), while eukaryotic cells (micrograph A3; red arrow) were pelleted. The removal of eukaryotic cells from the top fraction was confirmed using PCR (A4) with 16S rDNA primers (Bacteria and Archaea) and 18S rDNA primers (Eukarya). (T) = top fraction, (-) = negative control and (+) = positive control. Blastx analyses of the resulting metagenome was used to classify the sequences into taxonomic groups (the cutoff for this analysis was E-value <10-5) (B).

Table 2.1. Databases used in the analysis of the *Porites astreoides*-associated microbial metagenome. The database normalization option in blast was used in all analyses, however we have found that it is still necessary to use different E-value cutoffs based on the size of the database. SEED = SEED non-redundant database; Genbank virus database is at http://www.ncbi.nlm.nih.gov/genomes/static/vis.html. The SCUMS phage-prophage list is at http://scums.sdsu.edu/~rob/phage_nt.fasta; RDP = Ribosomal Database Project.

Taxonomical grouping	Database for BLAST comparison	No. of sequence similarities in the library	E-value cutoff
Bacteria	SEED	4,542	10^-5
Archaea	SEED	470	10^-5
Eukaryotic virus	Genbank	1,111	10^-4
Bacteriophage	SCUMS	1,984	10^-4
Fungi	SEED and RDP	25,026	10^-5
16S rDNA	RDP	387	10^-6
16S rDNA (mitochondrial)	RDP	503	10^-6
18S rDNA	RDP	47	10^-6
Mitochondria (coral)	Genbank	32,051	10^-5

The mitochondrial genome of Porites astreoides: The high occurrence of coral mitochondria DNAs (32,102 sequences total) in the metagenome provided enough sequence to reconstruct the coral mitochondrial genome. The large number of mitochondrial sequences was a consequence of our fractionation procedure for isolating the microbes associated with the coral (mitochondria are the same size as microbial cells). The cytochrome-oxidase C subunit 1 (*cox*1) gene, which is commonly used for phylogenetic comparisons [42], was assembled from the *Porites astreoides* metagenome and compared to *cox*1 from five other coral species. The phylogenetic analysis showed that *Porites astreoides* was most closely related to *Porites porites* (data not shown).

Coral-associated fungal communities: Fungi were the most abundant class of organisms represented in the *Porites astreoides* microbial metagenome. Members from three of the four fungal phyla (Ascomycota, Basidiomycota, and Chytridiomycota) were identified based on comparisons to the Ribosomal Database Project (RDP) (Maidak et al., 1996) and the SEED non-redundant database. The majority of coral-associated fungal sequences were most similar to Ascomycetes Fungi (93%) and therefore these were explored in more depth. Within the Ascomycetes, most of the Fungi were in the Class Sordariomycetes (77% for 18S rDNA and 71% for the SEED; Figure 2).

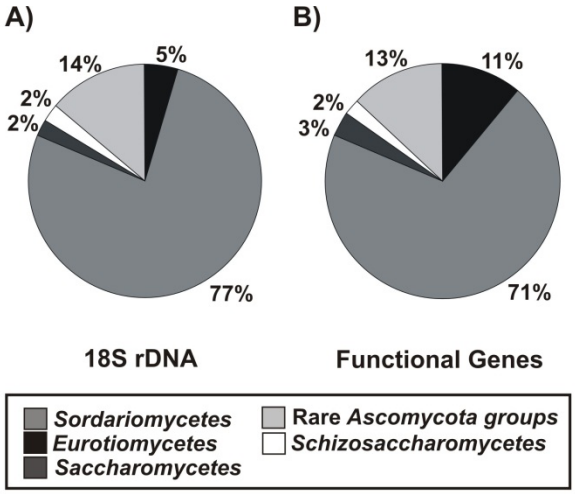

Figure 2.2. Composition of the coral-associated Ascomycetes Fungi community based on 18S rRNA (A) and the functional genes (B). The 18S rRNA assignments were determined based on comparisons to the Ribosomal Database Project (RDP) and functional genes were determined using the SEED non-redundant database.

Other fungal classes observed in the library include Eurotiomycetes, Saccharomycetes, and Schizosaccharomycetes. Previous microscopic studies have shown that endolithic fungi, classified as Acomycetes-like, are ubiquitously found across several genera of healthy corals [13, 43-45]. These Fungi associate with the

coral early in life and grow with the carbonate skeleton to maintain their position just under the coral tissue [44]. Corals recognize and defend their tissues against the endolithic fungi by encapsulating the advancing hypha through a dense layer of aragonite [43]. While endolithic fungi have not proven to be pathogenic, other Fungi, particularly *Aspergillus* spp., are known coral pathogens [46-48].

The metabolic potential of the fungal sequence similarities were analyzed using the SEED non-redundant database. Much of the coral-associated fungal genes encoded proteins involved in cell wall contruction (chitin synthase; number of sequence similarities (n) = 51), glutamate/aspartate/asparagine biosynthesis, glutamine metabolism (n = 65), and nitrogen metabolism, including the pathways for nitrate/nitrite ammonification (n = 21) and ammonia assimilation (n = 14; Table 2). These genes belong to multiple species, suggesting that the corals are harboring a diverse community of endolithic fungi.

Table 2.2. Functional genes in metagenome with significant similarities to Ascomycetes Fungi (based on an E-value $<10^{-5}$ to the SEED non-redundant database). Listed in the right-hand column are the proportion of sequences where the similarity was >90% to specific species.

Functional Gene	No. of hits	Subsystem	Similarities by family, >90%	Includes species	No. of hits
Chitin Synthase	51	Cell Wall Biosynthesis	Chaetothyriomycetes	*Exophiala dermatitidis*	3
			Dothideomycetes	*Phaeospaeria nodorum*	1
				Emericella nidulans	
				Aspergillus oryzae	
				Aspergillus fumigatus	
			Eurotiomycetes	*Penicillium chrysogenum*	9
				Coccidioides immitis	
				Coccidioides posadasii	
				Paracoccidioides brasiliensis	
			Leotiomycetes	*Botryotinia. fuckeliana*	3
				Fusarium oxysporum	
				Gibberella zeae	
			Sordariomycetes	*Magnaporthe grisea*	24
				Phialophora verrucosa	
				Glomerella graminicola	
				Neurospora crassa	
Nitrite Reductase	21	Nitrate/Nitrite Ammonification	Dothideomycetes	*Phaeospaeria nodorum*	3
			Sordariomycetes	*Gibberella zeae*	2
				Magnaporthe grisea	
Ammonium Transporter	7	Ammonia Assimilation	Pezizomycetes	*Tuber borchii*	1
			Sordariomycetes	*Gibberella zeae*	1
Ammonium Transporter Family	3		Sordariomycetes	*Gibberella zeae*	1
Uridylyltransferase	1		NA		
Nitrogen Regulation NR(I)	2		NA		
Sigma-54 factor rpoN	1		NA		
Glutamine Synthetase	5	Glutamate, Aspartate, & Asparagine Biosynthesis	Sordariomycetes	*Neurospora crassa*	1
			Eurotiomycetes	*Emericella nidulans*	1
			Pezizomycetes	*Tuber borchii*	1
Glutamate Synthetase [large subunit]	60		Saccharomycetes	*Saccharomyces cerevisiae*	3
			Schizosaccharomycetes	*Schizosaccharomyces pombe*	2
				Gibberella zeae	
			Sordariomycetes	*Magnaporthe grisea*	35
				Neurospora crassa	

Taxonomy of coral-associated Bacteria: The most prominent bacterial phyla in the metagenome were *Proteobacteria* (68%), *Firmicutes* (10%), *Cyanobacteria* (7%), and *Actinobacteria* (6%). These results are similar to a previous 16S rDNA analysis of *Porites*-associated Bacteria, where the same three phyla were the most dominant groups [1]. *Gammaproteobacteria* were the most abundant (31%) of all the bacterial sequences in the metagenomic library, once again showing striking similarity to the previous 16S rDNA analyses where 33% of coral-associated bacteria belonged to this group [1]. The most prominent genus was *Acinetobacter* sp. (n = 376). *Bdellovibrio* spp. (n = 206) were also frequently observed on the coral, raising the possibility that this group of bacteria may be important predators within the coral holobiont. The Cyanobacteria included representatives from four Orders, Chroococcales, which includes members of *Synechococcus* sp. and *Gloeobacter* sp., Nostocales, Oscillatoriales, and Prochlorales.

Metabolism of coral-associated microbes: It is now clear that metagenomes can be used to predict what microbes are doing in a particular ecosystem [38, 39], including the identification of novel metabolisms [49]. To characterize the metabolic potential of the Bacteria within the metagenome, these sequences were separated (E-value $<10^{-5}$) and compared to the functional genes within the SEED non-redundant database. Figure 3 shows 48 metabolic subsystems and their relative abundances within the coral-associated bacterial community. Overall, the community was heterotrophic and acquires carbon, sulfur, and nitrogen from the coral animal.

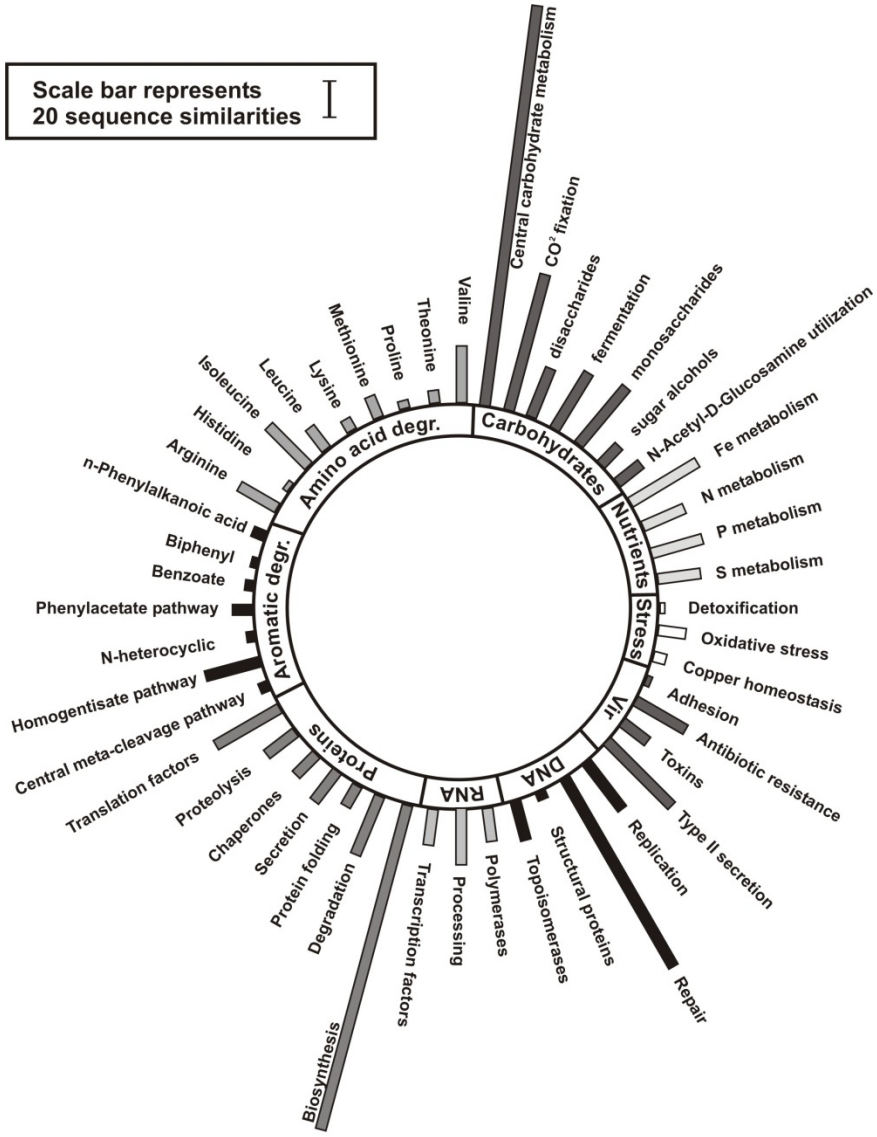

Figure 2.3. The metabolic potential of the coral-associated Bacteria within the metagenome. Sequences with best similarities to Bacteria (E-value <10-5) were classed into functional groups using SEED subsystems. Vir = virulence. Aromatic degr. = aromatic compound degradation.

Carbon metabolism of coral-associated microbes: The coral-associated bacteria possessed a large number of genes for the uptake and processing of sugars and proteins (Figure 3). Utilization pathways for sugars and proteins, as well as the associated transporters, were common in the metagenomic library (n > 250 sequences). In contrast, no transporters for the import of amino acids or fatty acids were observed (Figure 4A) Peptide transporters (n = 48) were in much higher abundance than sugar transporters (n = 12), but carbohydrate utilization pathways (n = 111) were more common than those for protein utilization (n = 30). Several pathways for the processing of glucose such as, glycosyl hydrolases and transferases were observed. Genes for the utilization of monosaccharides (n = 44) were higher than disaccharides (n = 30) and utilization of polysaccharides such as N-acetyl-D-glucosamine (n = 15) and sugar alcohols (n = 17) were also observed. Unlike the sugar and protein transporters, amino acid transporters were not observed, therefore the coral-associated microbes probably import the peptides and then recycle the amino acids inside the cell. In contrast, the common marine bacteria *Pelagibacter ubique*, predominately takes up amino acids from the environment [50]. The bacterial community has three times the number of genes for degradation of branched amino acids, such as, valine and isoleucine, than for other amino acids. In addition, pathways for the breakdown of amino acids with aromatic ring groups were more abundant. Specifically, the homogentisate pathway for degradation of aromatic compounds carries out the breakdown of L-phenylalanine and L-tyrosine (Figures 3 and 4A).

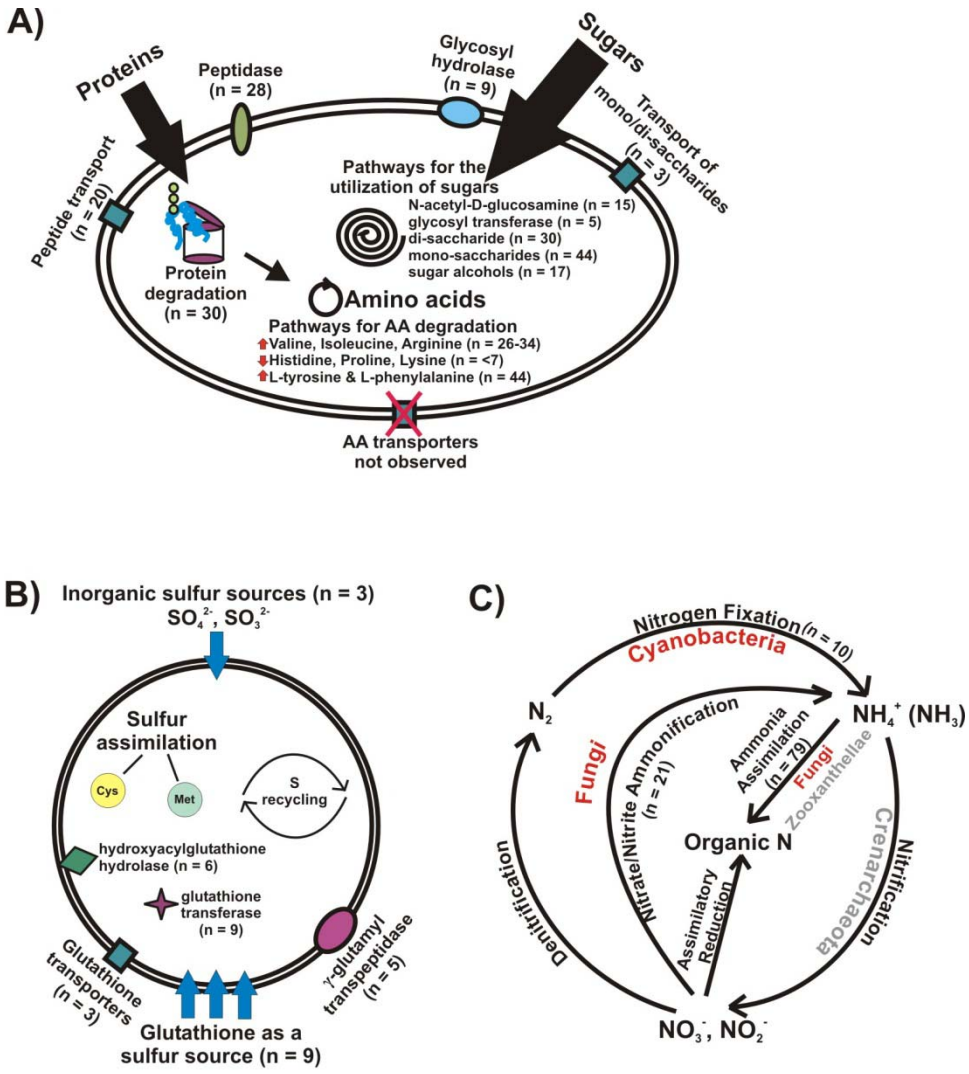

Figure 2.4. Proposed models for carbohydrate-protein (A), sulfur (B), and nitrogen (C) cycling in the Porites astreoides-associated microbial community. n = number of times a particular class of enzymes was found in the metagenome.

The microbial communities metabolic characteristics reflect the compounds found in coral mucus, where concentrations of proteins and polysaccharides are high, lipid content is lower, and amino acids are variable [51-53]. A study of the coral mucus content of six different species of corals showed that the mucus compounds were variable between all species [52]. It has been suggested that mucus of different

coral species enriches for different bacterial communities [23, 54, 55], and the community on the *Porites astreoides* coral has likely evolved to efficiently utilize the coral's mucus. Addition of poly- or mono-saccharides can disrupt this relationship and allow the coral-associated microbes to overgrow and kill the coral [56, 57].

Sulfur metabolism of coral-associated microbes: All living organisms require sulfur for the synthesis of proteins and essential co-factors. Both inorganic sulfur sources, such as sulfate, and organic sulfur sources are assimilated by microbes for the biosynthesis of amino acids, such as cystine and methionine. *Escherichia coli*, for example, uses glutathione in sulfur metabolism, redox regulation, and adaptation to stresses [58]. Genes for both the transport and degradation of glutathione were observed in the coral metagenome (n = 23). The abundance of genes for using glutathione as a sulfur source were three times higher than genes for assimilation of inorganic sulfur sources, such as sulfate (Figure 4B). Therefore, the coral-associated microbes are probably using glutathione as a sulfur source, which is abundant in eukaryotic cells [59].

Nitrogen metabolism of coral-associated microbes: Coral reefs often reside in oligotrophic waters and it has been suggested that the coral animal may acquire nutrients from their microbial counterparts. Though the coral animal is able to assimilate organic nitrogen either directly from the water column [60] or from their prey [61], efficiently recycling nutrients is still important and nitrogen-fixing microbes may subsidize the holobiont nitrogen budget. Several microbes potentially involved in nitrogen cycling have been identified within the holobiont and the current

metagenome confirmed their presence, as well as identifying new taxa (Figure 4C). Previously identified groups include Cyanobacteria [62-65], non-photosynthetic bacteria [65], and ammonia-oxidizing Crenarchaeotes [26, 49] (Figure 4C). It has been estimated that nitrogen-fixing Bacteria could provide up to 6% of the total nitrogen requirements of the coral community [65]. High numbers of Cyanobacteria cells (10^7 per cm^2) have also been observed on the surface of *Montastrea cavernosa* and the presence of nitrogenase proteins suggested these Cyanobacteria were capable of fixing nitrogen [63]. In the metagenomic library from *Porites astreoides* the Cyanobacteria accounted for 7% of the bacterial groups and included nitrogen-fixers, such as, Chroococcales and Nostocales. Nitrogenase (n = 6) and *nif* specific regulatory protein (n = 4) were also observed in the metagenome. Because the growth of zooxanthellae is nitrogen-limited [66, 67], it has been suggested that the symbiosis is dependent on the coral providing this nutrient [68], and these microbial symbionts could be important for providing fixed nitrogen. The endolithic fungi, described by the metagenomic library, were potentially involved in at least two processes of the nitrogen cycle within the coral (Figure 4C), including reduction of nitrate/nitrite to ammonia and assimilating ammonia for use in biosynthesis (Table 2), suggesting that the endolithic fungi play a previously undescribed role in the nitrogen cycle within the coral holobiont. Denitrification is the only nitrogen cycling process which has not been described in corals.

Stress and virulence genes in the metagenome: Oxidative stress response genes, were present in the metagenome (n = 16), suggesting that the community is

experiencing reactive oxygen conditions (Figure 3). This hypothesis is strengthened by the abundance of genes involved in DNA repair found in the library (n = 73). Coral-associated microbes have been shown to be exposed to high levels of ultraviolet radiation [5] and the metagenome suggests that coral microbes have repair mechanisms to deal with this stress.

The microbes associated with *Porites astreoides* coral have virulence genes (Figure 3). Several genes for the resistance of antibiotics and toxic compounds were observed (n = 34), specifically resistance to fluoroquinolones (n = 13) and toxic compounds cobalt, zinc, and cadmium (n = 19). Genes for the synthesis and secretion of toxins were also present in the library (n = 73). Taken together, these pathways suggest that the microbial community benefits from carrying genes for waging chemical warfare.

Coral-associated phage: Five phage (viruses that infect prokaryotes) families were observed in the microbial metagenome (Table 3). Microphage, the most abundant phage family, accounted for 51% of the phage similarities. These ssDNA phage were also abundant in other marine viriomes [69]. It was hypothesized that the abundances of these ssDNA viruses could be biased by the GenomiPhi DNA amplification procedure, however this has not been proven. Cyanophages made up 17% of all the phage sequences, suggesting that coral-associated phages may help regulate the microbial populations on the coral.

Coral-associated viruses have hosts that are mostly aquatic organisms: The eukaryotic viruses associated with *Porites astreoides* were identified by comparison to

the Genbank viral database (http://www.ncbi.nlm.nih.gov/genomes/static/vis.html; all sequences included in this section had an E-value < 10^{-4}). The viral species were then categorized at the family level. Most of the eukaryotic viruses found on corals were similar to those known to infect aquatic organisms (59%), such as fish, seabirds, crustaceans, algae, and protists (Table 3). The Iridoviridae are mostly known pathogens of aquatic organisms and they represented 37% of the viral similarities in the metagenome. Lymphocistis virus, which accounted for 17% of the coral-associated eukaryotic virus similarities, has been shown to infect many species of coral reef fish [70]. Lymphocistis virus causes a contagious, chronic infection leading to skin lesions in fish. While the disease is not lethal, it has been shown to reduce fitness of the host by affecting growth and increasing susceptibility to predation [71]. The identification of a high percentage of potentially pathogenic viruses on *Porites astreoides* suggests that the coral surface may act as a reservoir for pathogens. Corals have been previously implicated in spreading disease, for example, corals act as vectors for transmitting the digenean fluke, *Podocotyloides stenometra* to butterfly-fish [72]. Other marine animals have also been suggested to aid in the transfer of viruses to aquatic organisms, these include waterborne insect larvae [73], polychaete worms [74], and seabirds [75].

Table 2.3. Number of sequences in the metagenome with significant similarities to eukaryotic viruses and phage.

Virus family	Host	No. sequence similarities in library	% similarities to viral or phage database	Examples of observed taxa
		dsDNA viruses		
Baculoviridae	Crustacea, insects	23	2%	
Caulimoviridae	Plants	9	1%	
Herpesviridae	Molluscs, fish, mammals, turtles	354	32%	
Iridoviridae	Molluscs, fish, frogs	412	37%	*Lymphocystivirus* (LCDV), *Ranavirus*
Mimiviridae	Protozoa	105	9%	*Acanthameoba* mimivirus
Nimaviridae	Crustacea	5	1%	*Whispovirus* (white spot syndrome virus)
Phycodnaviridae	Algae, protozoa	71	6%	*Coccolithovirus* (EhV-86), *Ectocarpus* virus (EsV1)
Polydnaviridae	Insects	19	2%	
Poxviridae	Birds, crustacea, insects	26	2%	*Avipoxvirus* (canarypox and fowlpox virus)
Myoviridae	Bacteria	588	30%	Cyanophage, Aeromaonas and Vibrio phage
Podoviridae	Bacteria	52	3%	Cyanophage (P-SSP7)
Siphoviridae	Bacteria	226	11%	Mycobacterium phage
Unclassified	Bacteria, Archaea	47	2%	Psuedomonas and Actinoplanes phage
		ssDNA viruses		
Circoviridae	Birds	17	2%	*Circovirus* (beak and feather disease virus)
Nanoviridae	Plants	30	3%	Coconut foliar decay virus
Inoviridae	Bacteria	45	2%	Enterobacteria phage
Microviridae	Bacteria	1026	52%	Enterobacteria and Chlamydia phage

Water collected directly above four coral reefs in the Northern Line islands [76] were used to compare viruses on or within the coral to viruses found in the water column adjacent to the coral. The reef water was collected no more than five centimeters from the reef. Most of the viruses associated with coral (76%), were most similar to viral families that infect metazoans. In contrast, 61% of the viruses found in the reef water were most similar to families that infect algae and protozoa (Figure 5).

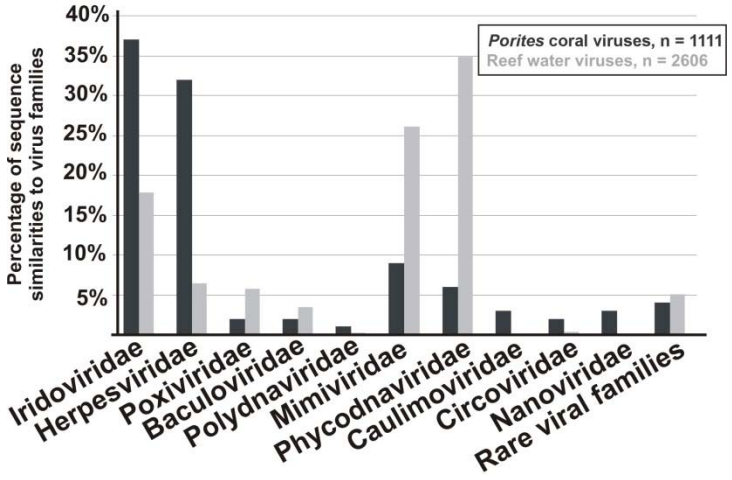

Figure 2.5. Comparison of viruses that infect eukarya found on *Porites astreoides* and in coral reef water. *P. astreoides* associated coral viruses are depicted by black bars and reef water viruses are depicted by gray bars. Similarities to eukaryotic viruses were determined by comparing the metagenomes to the viral genome list in Genbank (http://www.ncbi.nlm.nih.gov/genomes/static/vis.html; E-value cutoff used for this analysis was <10-4) and grouped into viral families.

How well did this approach work? While initially unanticipated, the co-purification of coral mitochondria with the other microbes was a useful way of obtaining taxonomic information on the animal host at the same time as the microbial constituents. Currently, coral taxonomy is in disarray [42] and this method would provide much needed data for better classification of corals (i.e., whole mitochondrial genomes).

PA1 is a Gammaproteobacteria that has been shown to be ubiquitously associated with *Porites astreoides* [1] and *Porites compressa* (Wegley, unpublished data). The current approach did not provide specific information on PA1. This may be rectified with more sequencing, but it would be nearly impossible to reconstitute the PA1 genomes from this mixture. Currently, it appears that the BAC/cosmid approaches would be best for constructing environmental microbial genomes [77].

The approach was very useful for generating hypotheses about potential roles of coral-associated microbes. In the future, we expect this approach will to be used to compare multiple metagenomes from different environmental and anthropogenic regimes using bioinformatics tools like XIPE [38] and cross-contig analyses [69].

Conclusions

The interactions of corals and microbes are one of the fundamental dynamics on reefs. Corals are increasingly faced with changing conditions on both regional and global scales. To understand how these changes will affect the coral holobiont, it is necessary to understand the roles of microbes and their responses to these changes. All of the current evidence suggests that corals and their microbial consortiums exist in a delicate balance [56, 57, 78], which can be disrupted such that microbes behave as specific and opportunistic pathogens (Harvell et al., 2007). Based on the microbial metagenome presented here, we suggest that the roles summarized in Table 4 are important to the functioning of the holobiont. There are almost certainly other important microbial-coral interactions still waiting to be uncovered in the current and

future metagenomes. The analyses and techniques presented here will provide another tool for determining how to these interactions change under different stressors with the goal of preventing coral holobiont collapse.

Table 2.4. Proposed roles of microbes inhabiting *Porites astreoides*.

Microbe type	Predicted functional roles
Endolithic fungi	Nitrogen cycling, including ammonification. Probably not living strictly parasitically as previously described.
Bacteria	Mostly heterotrophs, utilizing complex carbon compounds such as proteins and polysaccharides. Some carbon fixation and nitrogen fixation. Genes for stress response and virulence, in particular, DNA repair and antibiotic resistance appear to be important.
Eukaryotic viruses	The most abundant viruses present were most similar to viruses that infect coral reef fishes.
Phages	Preying on the bacterial community on the coral.

Materials and Methods

Coral Sampling: Porites astreoides samples were collected from Bocas del Toro, Panama (9˚ 19' 50" N, 82˚ 14' 58" W) using a punch and hammer. Coral fragments were placed in Ziploc bags, rinsed with sterile seawater at the surface and placed on ice until processed. Ten coral fragments (~2 cm^2) from different *P. astreoides* colonies were collected and subjected to the fractionation procedure. The resulting fractions were all assayed for the highest quality sample (described below).

Removal of zooxanthellae and coral animal cells: Coral fragments were diluted with PBSE, 1X phosphate buffered saline plus EDTA (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, and 10 mM EDTA) and ground into slurry using a mortar and pestle. Coral slurries were transferred to 15 ml centrifuge tubes and slowly spun in a table-top centrifuge at 100 × g to pellet the skeletal components. The supernatant was poured off into a glass homogenizer and mixed until the slurry appeared homogeneous (about 5 minutes). The coral slurry was then pressed through an 8.0 µm nucleopore filter (Whatman, UK) and separated into 2 ml polypropylene centrifuge tubes (four to six replicates were made per coral sample). Approximately 500 µl of coral slurry after the filtration step was stored at –20˚C for DNA isolation. Tubes were centrifuged at 500 × g at room temperature for 15 minutes. After this step the two fractions contained the following, the pellet consisted of coral nuclei, zooxanthellae, and other large entities and the supernatant consisted of mostly microbes and viruses. The pellet was rinsed twice with 1X PBSE, resuspended in 1 ml 1X PBSE, and fixed with 2% paraformaldehyde for microscopy. The supernatant

(containing microbes and viruses; Figure 1A) was poured off into a fresh 2 ml tube and spun at 8800 × g for 20 minutes at room temperature. The supernatant was again poured off and fixed with 2% paraformaldehyde for microscopy (this fraction was essentially free of larger organisms). The pellet was resuspended in 1 ml 1X PBSE and 500 µl of this solution was layered onto a cushion made of 15% Percoll (Fluka Chemika, Switzerland) in 1X PBSE. The coral solution was carefully layered using a sterile pipette onto a 1 ml Percoll cushion in a 2 ml centrifuge tube. Tubes were spun at 750 × g for 20 minutes at room temperature. The top layer (first 500 µl) contained almost solely microbial cells (Figure 1A), while the bottom fraction harbored the eukaryotic cells (Figure 1A). All top layers from the replicate tubes were collected using a plastic pipette with a cut off tip and combined (as were the bottom fractions). A small amount from both the top and bottom fraction were fixed with 2% paraformaldehyde for microscopy. The top and bottom fractions of each coral sample were stored at –20˚C until DNA isolation. All fractions, that had been fixed for microscopy, were stored at 4˚C.

DNA extraction and amplification: The top and bottom fractions of the gradient, as well as the coral slurry before centrifugation, were subjected to DNA extraction using the Ultraclean Power Soil DNA Extraction Kit (Mo Bio, Solano Beach, CA). In order to acquire sufficient concentrations (>1 µg/10 µl) for metagenomic sequencing, the DNA was amplified using φ29 polymerase (GenomiPhi; Amersham Biosciences, Pittsburgh, PA) [79]. DNAs from the top fractions were assayed for eukaryotic contamination using PCR. Primer sets used for this test

included the bacteria specific 16S rDNA gene (27F, 5'-AGAGTTTGATCMTGGCTCAG and 1492R, 5'-TACGGYTACCTTGTTACGACTT), the eukaryote specific 18S rDNA gene (EukA-F, 5'-AACCTGGTTGATCCTGCCAGT and EukB-R, 5-TGATCCTTCTGCAGGTTCACCTAC) and zooxanthellae specific primers (ZOOXss3Z, 5'-AGCACTGCGTCAGTCCGAATAATTCACCGG and ZOOXss5L, 5'-GGTTGATCCTGCCAGTAGTCATATGCTTG). The coral sample for metagenomic sequencing was chosen based on the quality and concentration of the DNA, the presence of a strong 16S band and absence of bands for 18S and zooxanthellae in the PCR reaction, and by visualization of the cells using epi-fluorescence microscopy. The selected DNA sample, which represented one coral fragment, was sequenced using pyrophosphate sequencing (454 Life Sciences, Inc., Branford, CT).

Bioinformatics: The metagenome sequences from each of the libraries were compared to the SEED non-redundant database using blastx [41, 80]. The SEED includes the GenBank database supplemented with other complete and draft genome sequences. The environmental database consists of the microbial assemblages from the Iron Mountain acid mine drainage [35], Sargasso Sea [81], whale fall [39], and Minnesota farm soil [39]. The contaminating sequences have been removed from the Sargasso Sea dataset [82, 83]. All large-scale computational analyses were performed on the Terraport and NMPDR cluster at Argonne National Laboratory and the Life

Sciences Gateway to the Teragrid. Individual analyses were performed on a 12-node Orion desktop cluster (Orion Inc., Santa Clara, CA).

These comparisons were supplemented with more extensive tblastn and tblastx comparisons [80] of either selected portions of the data against the complete non-redundant database or the whole library compared to boutique databases. The same cutoff E-value was always used for the same database and search method. In addition, the sequences were compared to the phage and prophage sequences from 510 genomes of the phage genome database and the 2323 genomes of the viral genome database (Edwards, unpublished). A FASTA file of these genomes is located at http://phage.sdsu.edu/oceanviruses/.

In an approach similar to previous work [37, 84], the best hit for each metagenomic sequence was automatically parsed and assigned as “known” if there was a significant hit ($E < 10^{-5}$) to a sequence from the non-redundant nucleotide database, else “unknown” (if there was no significant hit to any database). The number of hits in each group were then counted.

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CHAPTER 3: Taxonomic and metabolic diversity of coral reef-associated microbes across a latitudinal gradient

Coral-associated microbes are important members of a complex holobiont. Though it has been shown that these microbial symbionts perform beneficial functions to the host, few studies have characterized the biogeography and metabolic potential of these microbes across geographic space. To investigate the structural and functional differences of microbial communities associated with coral reefs traversing an expansive latitudinal gradient, metagenomes were constructed from the microbial communities associated with 26 coral reefs located in the central Pacific. The nutrient availability and percent cover of seven benthic functional groups measured at each metagenome site were used to determine which environmental predictors correlated with the microbial community structure or community metabolism. The distribution of microbial taxa was most strongly correlated with the prevalence of certain benthic functional groups. Reefs with higher coral cover observed on Islands Malden, Flint, and Vostok were associated with higher abundances of *Sphingomonadales*. In contrast, Kiritimati reefs dominated by turf algae (58.9-82.4%) had very little coral cover and were associated with higher abundances of *Bacteriodetes*. The reefs on Tabuaeran and Teraina had higher percent cover of macroalgae and were associated with greater abundances of *Gammaproteobacteria* (specifically, Orders *Pseudomonadales* and *Enterobacteriales*) and *Betaproteobacteria*. Regression analyses demonstrated that specific bacterial taxa were significantly correlated with different benthic functional groups. Bacteria from the Orders *Flavobacteriales* and

Alteromonadales were both positively correlated with the presence of turf algae on Line Island reefs ($r = 0.815$, $p = 0.002$ and $r = 0.682$, $p = 0.021$, respectively). The Genus *Synechococcus* was positively correlated with the presence of hard coral ($r = 0.665$, $p = 0.026$). The microbial community metabolism on LI reefs was most strongly influenced by geographic distance from the equator. Distance from the equator is strongly correlated with nitrogen and phosphorus concentrations suggesting that nutrient availability is an important driver for community metabolism on Line Island reefs. Metabolic pathways positively correlated with higher nutrients included conjugative transfer, chemotaxis, nitrate and nitrite ammonification, cobalt-zinc-cadmium resistance, multidrug resistance efflux pumps, and ton and tol transport. Low nutrient availability was correlated with metabolic pathways involved in photosynthesis, such as chlorophyll biosynthesis and photosystems I and II. The results from this study suggest that selection of microbial taxa is based on carbon sources (benthic community composition) and subsequently, specific genes are incorporated for adaptations to nutrient availability in that region.

Introduction

Genomic studies of marine microbes highlight the vast array of metabolisms available for acquiring energy and nutrients. This research has also provided a greater understanding of community ecology (i.e., biogeography) and the evolution of species (e.g., species level genomic variation commonly referred to as the pan or mobile genome). Surface marine bacterioplankton are predominately composed of cosmopolitan microbes with streamlined genomes that promote slow, steady growth and lack the ability to respond quickly to nutrient pulses (e.g., *Pelagibacter ubique* and high light ecotypes of *Prochlorococcus marinus*) [1-3]. Bacterioplankton community structures are also characterized by a long tail of less abundant to rare members adapted to respond quickly to nutrient pulses and likely inhabit particulates in the water column such as marine snow or the surfaces of eukaryotic plankton [4, 5]. Metagenomic investigation of bacterioplankton in the deep ocean demonstrated that the rare members observed in surface plankton communities become more prevalent in the cold and deep biosphere. In this ecosystem, genomic adaptations constitute larger genomes with greater metabolic versatility and plasticity facilitating the utilization of a diverse array of metabolic substrates should they become available [6]. Though some studies have reported genomic characteristics of microbes that reflect the nutrient availability of the region [7, 8] most of this data is demonstrated in the genomes of representative members rather than the community metabolism as a whole.

Microbes overlying or within the boundary layer of coral reefs share attributes of both pelagic and benthos-associated communities. Common members of pelagic

bacterioplankton communities, such as unicellular cyanobacteria and *Pelagibacter ubique* are observed, however rarer members in the surface plankton, such as Proteobacteria and Firmicutes are more abundant. The composition of reef microbes can also reflect ecosystem health. Degraded coral reefs where fleshy algae dominates the benthos are characterized by higher microbial abundances and compositions dominated by copiotrophics, many of which are known pathogens [9]. Community metabolism also differs substantially on degraded reefs reflecting the heterotroph-dominated system [9] as well as an increased ability for pathogenesis as suggested by the increased prevalence of virulence genes [10]. Reef microbes present in the boundary layer also likely reflect those inhabiting the benthic organisms. Coral-associated bacteria have been shown to be highly diverse and species specific [11]. Though less characterized, there is also evidence that specific bacterial taxa are associated with certain algal functional groups [12].

In this study, we constructed metagenomes from the microbes associated with 26 coral reefs to investigate community differences between reefs on uninhabited versus populated islands and the influence of biogeochemistry on microbial community structure and metabolism.

Materials and Methods

Sample collection. For metagenomes, approximately 100 l of seawater was collected from below the benthic boundary layer (in crevices and against the benthos) across ~20 m^2 of reef using a modified bilge pump connected to low density polyethylene (LDPE) collapsible bags (19 l; Cole-Parmer, Vernon Hills, IL, USA) and

transported to the research vessel within two hours. Prior to sampling, containers, bilge pumps, and tubing were washed once with 1% bleach and 0.1 N NaOH, three times with freshwater, and once with 100 kDa filtered seawater. Large eukaryotes were removed by filtration through 20 μm Nitex. The filtrate was then concentrated to <500 ml using a 100 kDa tangential flow filter (TFF), which captured the unicellular eukaryotes, microbes, and virus-like particles. Microbial cells were collected by passing the concentrated sample through 0.45 μm Sterivex filters (Millipore, Inc) and the filters were stored at -80 ^{o}C.

Water for nutrient analysis was collected directly overlying the reef (within 20 cm of the benthos) using diver deployable polycarbonate Niskin bottles. Water samples were filtered through 0.2 μm Nuclepore Track-Etch membrane filters (Whatman, Kent, U.K.) into 20 ml HDPE scintillation vials with cone-shaped plastic lined lids (Fisher Scientific, Massachusetts, USA). Samples were then stored at -20^{o}C until analyzed. Analysis of inorganic nutrient (nitrate, nitrite, ammonium and phosphate) concentrations was carried out by the Marine Science Institute Analytical Lab at University of California, Santa Barbara, CA using a QuikChem 8000 flow injection analyzer (Lachat Instruments, Wisconsin, USA).

DNA extraction and metagenomic library construction. DNA was extracted and purified using a modified column purification protocol (Nucleospin Tissue, Macherey-Nagel, Dueren, Germany). Lysis buffer (T1 solution, 360 μl) plus 50 μl proteinase K (20 mg/ml) was added to each Sterivex filter (closed on both ends with luer lock fitted caps). Sterivex filters plus lysis buffer were rocked at 55^{o} C for 12

hours. Buffer B3 (400 µl) was added to Sterivex filters and incubated for 15 minutes at 70^{o} C. Lysed DNA solution was removed from the Sterivex using a 3ml syringe with luer lock fitting and placed into eppendorf microfuge tube. 100% ethanol was added (400 µl) and vortexed, then lysis solution was loaded onto filter columns. The remaining extraction procedure was followed as recommended by the manufacturer. Metagenomic libraries were prepared using the GS FLX Titanium Rapid Library Preparation Kit (Roche Applied Sciences, Connecticut, USA) and pyrosequenced using the 454 GS-FLX platform.

Sequence library quality control and bioinformatic analyses. Metagenomic sequences were QC processed using Prinseq [13] and uploaded to the MG-RAST server (http://metagenomics.nmpdr.org/metagenomics.cgi) for functional and taxonomic annotation. Sequences were compared to the SEED protein database using BLASTx [14]. Sequences with significant similarities (E-value <0.00001) were assigned functions based on their closest similarity [15] and were then grouped into metabolic pathways (subsystems).

Statistics. Statistical analyses were performed using R 2.15.1 (R Development Core Team, http://www.r-project.org) [16] unless otherwise denoted. Non-metric multidimensional scaling (nMDS) analyses were used to visualize between-island similarity in terms of two discrete response variables: taxonomic structure and community metabolism. nMDS analyses were completed using the isoMDS function with a Euclidean distance matrix or in rare cases, a Manhattan distance matrix. Significant groupings of islands were determined using a similarity profile test based

on the Bray-Curtis algorithm (SIMPROF) [17] using the clustsig package (http://CRAN.Rproject.org/package=clustsig) [18] and 10,000 random permutations of the raw data. These significant groupings were then superimposed upon the MDS plots. A canonical correspondence analysis (CCA) was used to investigate correlations with the predictor variables using the Vegan package (http://CRAN.R-project.org/package=vegan) [19]. The correlation between island position in multivariate space and the correlation with the predictor variables was visualized using the CCA by calculating and plotting the loading vectors. The CCA should not be viewed as a form of formal hypothesis testing; we present the correlations between taxa abundance/metabolism and all predictor variables purely for data exploration. To formally quantify the proportion of variation in taxa abundance/metabolism across islands explained by the predictor variables, a permutational distance-based multivariate linear model (DISTLM) [20] was used in PERMANOVA+. Predictor variables were first investigated for collinearity by calculating Pearson's correlations between each pair. No two predictors exceeded a correlation of 0.75 and therefore all were included in the model (Supplementary Table 1). Model selection was based on Akaike's information criterion [21] with a second-order bias correction applied (AICc) [22]. Models were run on 10,000 random permutations of the raw data. Finally, the program Xipe [23] was used to determine finer taxonomic groups and metabolic pathways that were significantly different (p-value <0.05, 1000 iterations) between metagenomic libraries. To determine whether specific taxonomic groups and metabolic pathways identified by Xipe analysis correlated with nutrient availability or

benthic functional groups across all eleven LI, linear regression analyses were plotted using Sigma Plot 8 (Systat software, Inc., San Jose, CA) and Pearson correlation coefficients were calculated using SPSS (IBM Corporation, Armonk, NY, USA).

Results and Discussion

Natural history of the Line Islands. The Line Island (LI) archipelago consists of eleven atolls spanning a latitudinal gradient from 6^{o} north to 11^{o} south. Nutrient concentrations vary across the islands where inorganic nutrient concentrations are approximately five and two-times higher for nitrogen and phosphorus, respectively on Jarvis (located closest to the equator) compared to Kingman and Flint (located furthest north and south) (Figure 1, Supplementary Table 2). Despite higher nitrogen levels than many oceanic regions, the reefs surrounding the LI are characteristic of oligotrophic environments. Iron is extremely limited in the region which is referred to as a high nitrate low chlorophyll system [24]. The reef ecosystems on the LI have high cover of stony corals (mean = 44.4%, max = 87% and min = 2%, Supplementary Table 2) and large biomass of fishes [25]. Three of the 11 LI have small human populations (approximately1000, 2,500, and 5,000 people live on Teraina, Tabuaeran, and Kiritimati, respectively). Reefs on the populated islands are subjected to subsistence and commercial fishing, as well as slight levels of pollution and runoff. Because of their remote location, the uninhabited islands are largely spared of anthropogenic disturbances such as commercial fishing and agricultural runoff. One site on Moorea, French Polynesia and three sites on Curacao, in the Caribbean were

also included to investigate regional differences in coral reef associated bacterial communities.

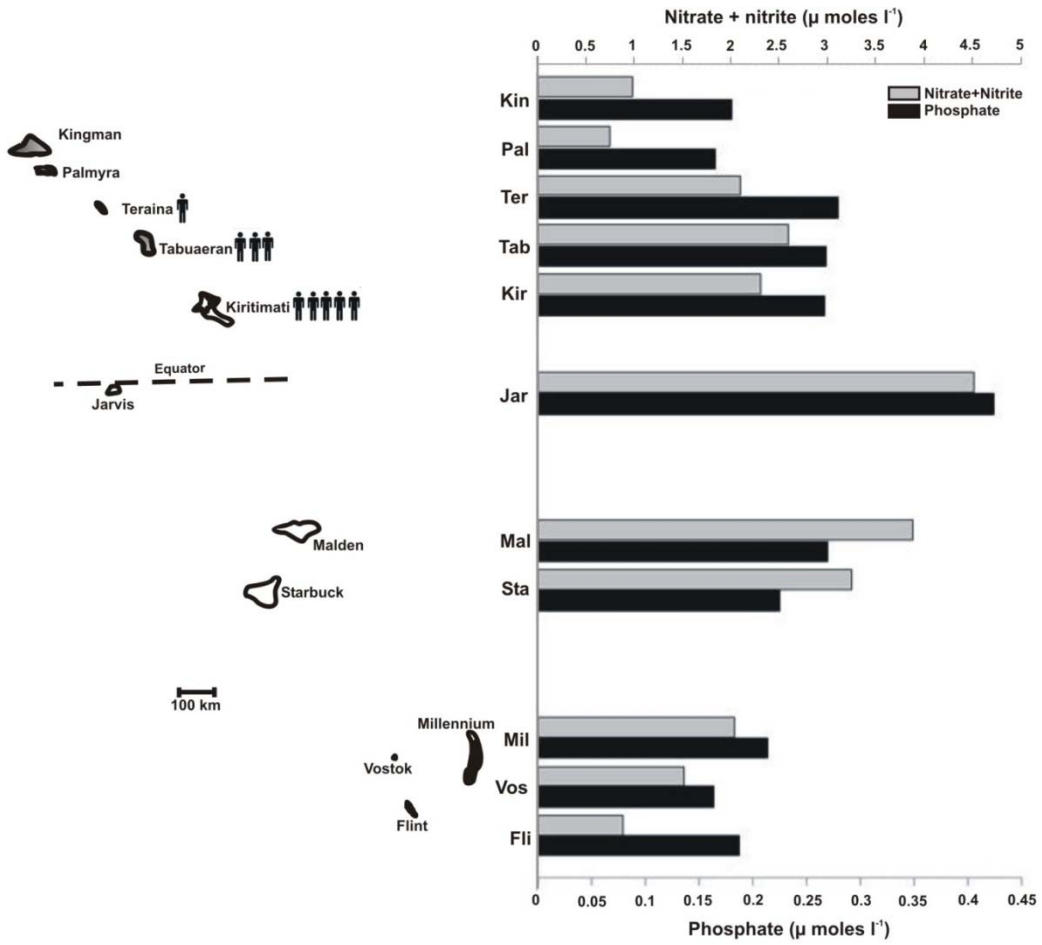

Figure 3.1. Map of the Line Islands and the nutrient concentrations. The scale bar corresponds to distance between islands. Island sizes are not to scale though are depicted in relative proportion to one another. Human populations are denoted by symbols. Each human symbol represents approximately 1000 individuals. Nutrient concentrations were measured in triplicate for each metagenome site. The bar graph represents averages of replicates per site and sites per islands. For numeric values and standard error calculations for nitrate and phosphate measurements, see Supplementary Table 1.

Table 3.1. Sample metadata and metagenomic library details

Sample name	Date collected	Total no. sequences	Avg. seq length (bp)	LAT	LONG	% GC	Total no. bacterial similarities	Total no. subsystems similarities
Curacao 1	9/30/2009	32708	357.67	12.122946	-68.97147	38	14268	19022
Curacao 2	10/1/2009	27933	349.1	12.100948	-68.92708	38	12046	16042
Curacao 3	10/21/2009	26657	357.1	12.369642	-69.15506	37	12633	16581
Flint 2	3/30/2009	24111	345.71	-11.41924	-151.82739	51	8454	10167
Flint 5	3/29/2009	98284	399.55	-11.43911	-151.81964	47	28931	37301
Flint 6	3/31/2009	39070	430	-11.44423	-151.81709	47	17316	20252
Jarvis 4	4/4/2010	171749	400.17	-0.38188	-159.99800	48	49941	61987
Jarvis 9	4/2/2010	235984	407.55	-0.36537	-160.00600	50	63207	77340
Jarvis North	11/13/2010	66808	384.81	-0.36902	-160.00819	52	20340	23781
Jarvis Tent	11/12/2010	49774	397.8	-0.369017	-160.00819	50	22465	24945
Kingman 2	10/31/2010	225914	381.44	6.387	-162.38600	52	47187	61909
Kiritimati Oil	11/21/2010	156251	393.74	1.99095	-157.48251	51	54015	62427
Kiritimati Tent	11/20/2010	30131	387.29	2.0085833	-157.48945	50	11457	12895
Malden 25	4/11/2009	164564	381.72	-4.03326	-154.95094	52	42411	51614
Malden 5	4/10/2009	48258	349.25	-3.99531	-154.94452	57	10993	12902
Millennium 12	4/19/2009	26895	357.99	-9.90774	-150.19974	53	7801	9190
Millennium 9	4/17/2009	39933	373.89	-9.91672	-150.21072	54	13032	15772
Moorea	9/15/2010	49062	377.76	-17.48642	-149.84556	42	13694	16999
Palmyra 1	10/25/2010	170135	386.57	5.86646	-162.11346	37	53623	71606
Starbuck 13	4/5/2009	29347	401.56	-5.66441	-155.87346	46	11874	15052
Starbuck 7	4/6/2009	83014	431.87	-5.62220	-155.88002	42	34058	45652
Tabuaeran 10	11/4/2010	104845	396.09	3.82595	-159.34957	56	39697	46930
Tabuaeran 2	11/6/2010	73712	411.89	3.84085	-159.36047	55	31874	36241
Teraina 2	11/9/2010	42317	385.46	4.70242	-160.39212	53	12035	14199
Teraina Tent	11/8/2010	285841	412.73	4.6867167	-160.42023	51	86972	101107
Vostok 10	4/1/2009	83219	357.47	-10.05835	-152.30954	44	32232	41533
Total	**-**	**2386516**	**-**	**-**	**-**	**-**	**752556**	**923446**

No., number. Avg., average. Seq., sequence. bp, base pairs. LAT, latitude. LONG, longitude.

Comparative metagenomics. Metagenomes from 26 reefs comprising 22 from the LI, one from Moorea, French Polynesia, and three from the Caribbean (Curacao, Netherlands) were compared using BLASTx (Alschultz et al., 1990) with evalue < e^{-05} using the MG-RAST server [15]. There were a total of 2.39 million sequences after quality control using Prinseq [13] with an average read length of 389 base pairs (Table 3.1).

Biogeography of microbial communities across the Line Islands. The distribution of taxa on LI reefs did not group based on geographic distance. For example, the two most northern LI, Kingman and Palmyra group with the Southern LI (Group 1, Figure 3.2). Within Group 1, Kingman and Palmyra were most similar to Millennium, one of the most southern islands. Likewise Malden and Flint are separated by nearly 900 km, but had similar taxonomic distributions. Group 1 comprises all the uninhabited LI and is driven by higher abundances of *Cyanobacteria* and *Alphaproteobacteria* (i.e., Orders *Rhodobacterales* & *Rickettsiales*) and *Firmicutes*. Teraina, Tabuaeran, and Kiritimati, which represent the three populated LI, showed the greatest differences in regards to taxa and Kiritimati formed a separate outgroup by itself (Supplementary Fig. 1). Teraina and Tabuaeran (Group 2) had higher abundances of *Gammaproteobacteria* (specifically, the orders *Alteromonadales*, *Vibrionales*, *Enterobacteriales*, and *Pseudomonadales*) and *Betaproteobacteria* (Figure 3.2). Kiritimati (Group 3) had high abundances of *Bacteriodetes* (25.1% ±4.2%, N=2) compared to average abundances on other islands (7.2%±3.5%, N=20, Supplementary Fig. 2).

The distribution of metabolic genes showed greater similarity amongst LI in close proximity to each-other. Kingman and Palmyra formed a group that was driven by higher abundances of cofactors, RNA metabolism, and protein metabolism (Figure 3.2). Jarvis, Kiritimati, Teraina, and Tabuaeran grouped together with Jarvis and Kiritimati being the most similar amongst all four islands for the relative abundance of metabolic genes (Supplementary Fig. 3). These islands were characterized by higher

abundances of metabolic genes for aromatic compound utilization, iron metabolism, membrane transport, nitrogen metabolism, potassium metabolism, regulation, and virulence. The remaining five southern LIs, Flint, Malden, Millennium, Starbuck, and Vostok grouped together in the center of MDS plot with the northern-most island, Malden being less similar than the other four (Figure 3.2 and Supplementary Fig. 3).

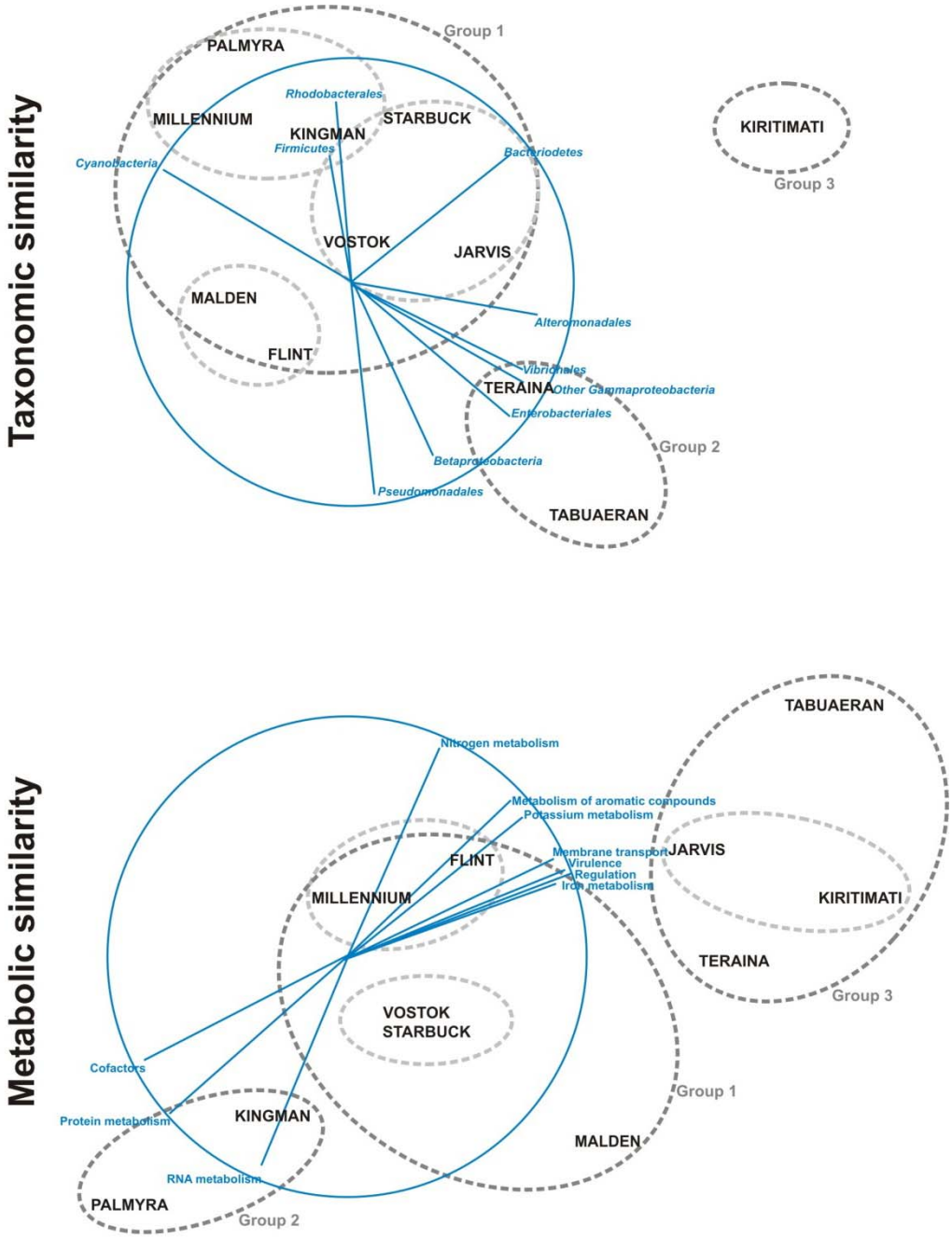

Figure 3.2. Multidimensional scaling plots for the relative abundances of taxonomic similarities (top) and metabolic subsystem similarities (bottom). Sites were pooled to create island means. The 2D stress values for the two plots are 0.05 and 0.03 for the taxonomic similarities and the metabolic similarities, respectively. The dark gray circles represent the significant groupings generated by the SIMPROF analysis. The light gray circles represent clusters of islands that were more similar to each other but not significantly different.

Predictor variables correlated with microbial community structure and metabolism. The nutrient availability and percent cover of benthic functional groups measured at each metagenome site were used to determine whether certain predictor variables correlated with the microbial community structure (taxonomic similarities) and the community metabolism (metabolic similarities) on LI reefs. Benthic functional groups were classified as hard coral, crustose coralline algae (CCA), other calcified macroalgae, soft coral, macroalgae (fleshy), turf algae, and other benthic organisms (Supplementary Table 3 provides a detailed list of the genera included in each category). The other predictor variables used included the concentrations of inorganic nutrients (nitrate + nitrite and phosphate) and distance from the equator.

A canonical correspondence analysis (CCA) was used to visualize the correlations between the island position (based on the relative proportions of taxonomic or metabolic similarities) and the predictor variables. The LI reefs with higher coverage of hard coral (Group 1 islands, such as Malden and Flint) correlated with higher abundances of *Alphaproteobacteria*, *Cyanobacteria*, and *Firmicutes* (Figure 3). In contrast, Kiritimati reefs, which were dominated by turf algae (58.9-82.4%) and had very little coral cover, were associated with higher abundances of *Bacteriodetes*. The reefs on Tabuaeran and Teraina had higher abundance of fleshy macroalgae and were associated with greater abundances of *Gammaproteobacteria* and *Betaproteobacteria*. Hard coral alone formed the optimal model for microbial community structure on LI reefs based on the DISTLM, explaining 15.2% of the variation (Table 3.2). Taken together, the DISTLM and the correlations depicted by

the CCA suggest that microbial community structure on LI reefs correlated best with benthic community composition.

Geographic location and nutrient availability correlated better with the microbial community metabolism than with community structure. Higher concentrations of phosphate and nitrate were correlated with Jarvis, Tabuaeran, and Teraina (Figure 3.3). Geographic location, measured as latitudinal distance from the equator, and coral cover correlated with southern LI Millennium and Flint. The DISTLM demonstrated that distance from the equator provided the optimal model for metabolic gene similarities explaining 18.4% of the variation (Table 3.2). Thus, islands in close proximity to each-other tended to have similar microbial community metabolism. The reef associated microbes located in a similar geographic context had similar community metabolism despite differences in the distribution of taxa present a reef sites. For example, Jarvis and Kiritimati had similar community metabolism, whereas Jarvis was most similar to Vostok and Starbuck for microbial community structure and Kiritimati's microbial community was the most different of all the LI. Therefore, microbial communities composed of different taxa are still performing similar functional roles.

Table 3.2. Summary results of a distance-based permutational multivariate multiple regression model (DISTLM) for associations of microbial community structure (Taxa) and metabolic function (Metabolism) with the percent cover of benthic functional groups, distance from the equator and nutrient availability (total number of predictors included = 10). The optimal models are shown, along with the proportion of variability in the multivariate response variable explained (Prop.)

Variable	AICc	SS (trace)	Pseudo-F	P	Prop.	res.df
			Taxa			
Hard coral	129.52	1438.1	3.4065	0.0215	15.2%	19
			Metabolism			
Distance from equator	53.323	47.953	4.2767	0.0147	18.4%	19

AICc, Akaike's information criterion. Prop., proportion of variance. res.df, degrees of freedom

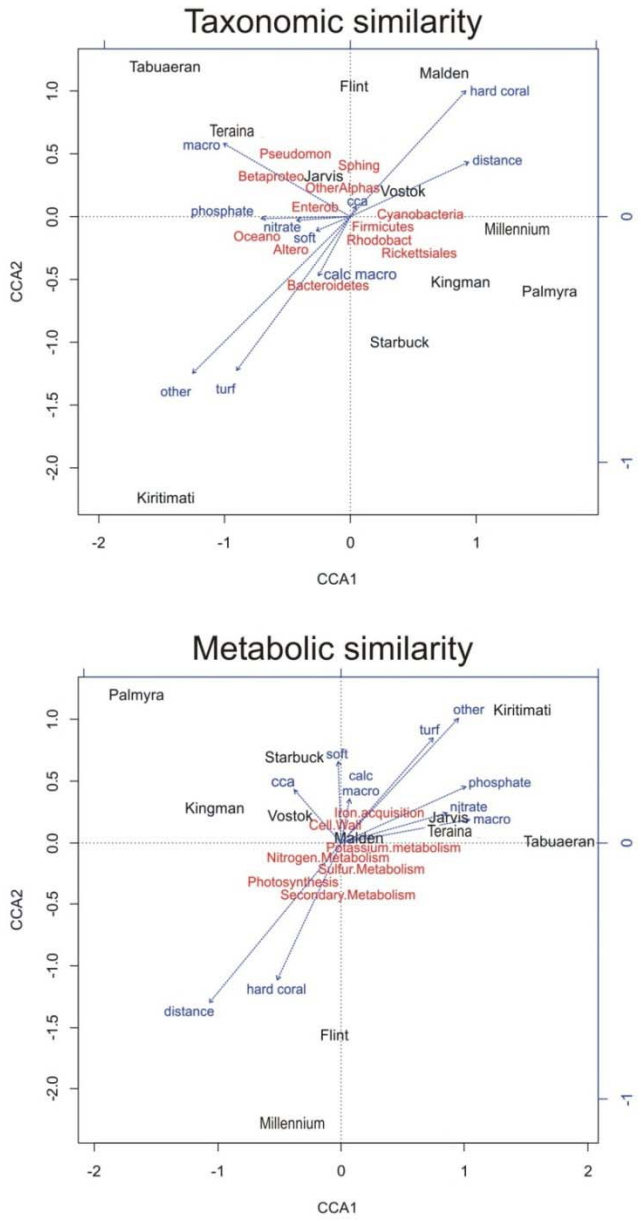

Figure 3.3. Canonical correspondence analysis (CCA) depicting the correlations between environmental predictor variables (blue) and the relative abundance of taxonomic similarities (A) and metabolic similarities (B) on each Line Island. Loading vectors for the taxa and subsystems are shown in red. Altero, *Alteronmonadales*. Betaproteo, *Betaproteobacteria*. Enterob, *Enterobacteriales*. Oceano, *Oceanospirillales*. OtherAlphas, Other *Alphaproteobacteria*. Pseudomon, *Pseudomonadales*. Rhodobact, *Rhodobacterales*. Sphing, *Sphingomonadales*. calc macro, other calcified macroalgae. cca, crustose coralline algae. dist, distance from the equator. macro, macroalgae (fleshy). other, other benthic organisms. soft, soft coral.

The question remains as to what environmental parameters associated with geographic location are driving the community metabolism of reef microbes. Nutrient availability was thought to be an important variable because of the well-established oceanic nutrient regimes where upwelling brings higher concentrations of inorganic nutrients to surface waters near the equator. Distance from the equator was correlated with both phosphorus and nitrogen concentrations ($r = -0.74$ and -0.64, respectively, Supplementary Table 1), though neither exceeded the cutoff value for collinearity so they were not excluded from the model. Phosphate and nitrate were not as strong of predictors as latitudinal distance from the equator and their inclusion in the linear model did not produce the best fit. It is possible that distance from the equator is a better predictor than the nutrient concentrations themselves because of under sampling. Island averages of 20+ sites might have produced better fit, than the few sites collected from each metagenome site. Furthermore, other parameters associated with geographic location such as seawater temperature, salinity, PAR, or availability of micronutrients (e.g., iron) could also be influencing microbial community metabolism and therefore may contribute to this variable providing the better fit. Nevertheless, the availability of macronutrients nitrogen and phosphorus are posited to be important influences on microbial community metabolism on LI reefs.

Specific metabolic pathways and microbial taxa correlated with predictor variables. The sample size of metagenome sites (N =22) limits the resolution we can compare taxonomic and metabolic sequence similarities using multivariate statistical analyses. For these tests, only the coarsest categories can be used (level one subsystems in the SEED database, e.g., amino acid metabolism). The program Xipe [23] uses a bootstrapping technique to allow comparison of thousands of gene categories between two metagenomic libraries with a designated confidence threshold (e.g., 95%). Xipe was used as an exploratory tool to investigate which metabolic pathways (level 3 subsystems, e.g., alanine synthesis) and microbial taxa (i.e., Genus rank) were overrepresented on different LI. Metabolic pathways and microbial taxa identified using Xipe were tested for significant correlations with different predictor variables across all eleven LI.

Specific bacterial taxa correlated with different benthic functional groups. The CCA and Xipe analysis provided insight into specific taxonomic groups of bacteria that may be associated with different benthic organisms. Bacteria from the Orders *Flavobacteriales* and *Alteromonadales* were both positively correlated with the presence of turf algae on reefs (Table 3.3, $r = 0.815$, $p = 0.002$ and $r = 0.682$, $p = 0.021$, respectively). There was a positive correlation between the Class *Gammaproteobacteria* and the presence of macroalgae, although this was not significant ($r = 0.560$, $p = 0.073$). The Genus *Synechococcus* was positively correlated with the presence of hard coral ($r = 0.665$, $p = 0.026$). This result was surprising as the relative abundances of unicellular *Cyanobacteria* are often associated with nutrient

concentrations. As such, *Prochlorococcus* abundances were associated with oligotrophic conditions, correlating negatively with higher phosphate concentrations ($r = -0.614$, $p = 0.045$). There was a strong negative correlation between hard coral cover and abundances of *Alteromonadales* ($r = -0.819$, $p = 0.002$), suggesting that corals and / or their associated microbial symbionts may inhibit the growth of these species.

Table 3.3. Significance test for the linear correlations of specific taxa and environmental parameters.

Taxa	Environmental parameter	Pearson's coefficient r	p value
Flavobacteriales	Turf algae	0.815	**0.002**
Alteromonadales	Turf algae	0.682	**0.021**
Alteromonadales	Hard coral	-0.819	**0.002**
Synechecoccus	Hard coral	0.665	**0.026**
Gammaproteobacteria	Macroalgae	0.560	0.073
Prochlorococcus	Phosphate	-0.614	**0.045**
Sphinogomonadales	Nitrate	0.758	**0.007**
Erythrobacter	Nitrate	0.674	**0.023**

The taxonomic distribution of bacteria demonstrated here may reflect the symbiotic community directly associated with the different benthic functional groups. Corals thrive in nutrient limited regions because of a mutualistic association with the unicellular algae, zooxanthellae. This coral holobiont extends to include other beneficial microbes, notably the coral-associated bacteria, which have been shown to be species specific [11, 26-28], increase nutrient availability [29-32], and suggested to inhibit infection by pathogens [33, 34]. Barott and colleagues [12] found that specific bacterial OTUs were associated with groups of benthic algae, suggesting that algae

may also exist as a holobiont where their microscopic counterparts confer selective advantages.

Alternatively, these bacteria may be selected based on the organic compounds exuded by the benthic community as microbes collected for this study reflect both those living on and within the diffusive boundary layer of the reef. An empirical study by Nelson and colleagues [35] demonstrated that coral and fleshy macroalgal exudates enrich for distinct bacterioplankton communities. Coral exudates selected for several lineages of *Alphaproteobacteria* including *Erythrobacteraceae* whereas exudates released by fleshy macroalgae selected for *Gammaproteobacteria* lineages including members from the families *Alteromonadaceae*, *Psuedoalteromonadaceae*, and *Vibrionaceae* [35]. In this study, the *in situ* microbial communities on reefs with high coral cover were also associated with higher abundances of *Alphaproteobacteria* (Figures 2A and 3A). *Erythrobacter* spp. were also overrepresented on high coral cover reefs of Malden (N=3 Xipe comparisons, $p < 0.05$), though these taxa did not correlate with percent coral cover across all LI. Reefs on Tabuaeran and Teraina which both had high cover of fleshy macroalgae were correlated with some of the same groups of *Gammaproteobacteria* highlighted by the Nelson et al. study, namely *Alteromonadales* and *Vibrionales* (Figure 3.2). In addition, five *Bacteriodetes* genera *Croceibacter*, *Dokdonia*, *Gramella*, *Leeuwenhoekiella*, and *Polaribacter* (all of which are *Flavobacteria*) were consistently overrepresented on turf dominated Kiritimati compared to sites on other islands (N=3 Xipe comparisons, $p<0.05$, data not shown). Three of the same *Bacteriodetes* genera (i.e., *Dokdonia*, *Gramella*, and

Leeuwenhoekiella) were significantly overrepresented in bacterioplankton communities enriched with turf algal exudates. Together, these results suggest that coral and algal-derived organic exudates enrich for specific types of bacteria and that these enrichments can be observed on *in situ* reef communities.

Specific metabolic pathways correlated with nutrient availability. Nine pathways demonstrated positive or negative correlations for the eleven LI when compared with phosphate concentrations. Metabolic pathways positively correlated with higher nutrients include conjugative transfer, chemotaxis, nitrate and nitrite ammonification, cobalt-zinc-cadmium resistance, multidrug resistance efflux pumps, and ton and tol transport (Figure 3.4). Two subsystems involved in membrane transport, conjugative transfer and ton and tol transport, were positively correlated with higher phosphate ($r=0.863$, $p=0.001$ and $r=0.650$, $p=0.03$, respectively, Table 3.4). The conjugative transfer subsystem includes genes involved in type IV secretion of proteins and nucleoproteins. These genes may be important for central metabolism, such as for the secretion of ectoenzymes and sideophores for energy and nutrient acquisition. Overrepresentation of the conjugative transfer subsystem also suggests that horizontal gene transfer may play a larger role at high nutrient sites. TonB-dependent transport is used to transport large, complex molecules through the outer membrane. Substrates include dissolved organic matter (polysaccharides, proteins, and organic phosphorus) and iron chelates (siderophore and heme iron complexes). These transporters have been shown to be important in marine environments as demonstrated by their presence in marine bacterial genomes and pelagic metagenomes [36, 37], high

levels of expression in metatranscriptome data [38] and their predominance amongst all membrane proteins identified by proteomic analysis of pelagic bacteria. Our results suggest that higher nutrient availability selects for these transporters (nearly 1% of gene similarities at some high nutrient sites) compared to oligotrophic islands. Two subsystems comprising genes for resistance to antibiotics and toxic compounds (i.e., cobalt-zinc-cadmium resistance and multidrug resistance efflux pumps) were correlated with higher phosphate ($r=0.637$, $p=0.035$ and $r=0.617$, $p=0.043$). The genes might play a role in nutrient acquisition, but may also be important mechanisms to defend against chemical warfare from other microbes as well as the benthic algae. Nitrate and nitrite ammonification also referred to as dissimilatory reduction of nitrate to ammonium (DRNA) was present in higher abundances on high nutrient reefs ($r = 0.628$, $p = 0.038$, Table 3.4). DRNA is an anaerobic process which may be an important nitrogen metabolism in the diffusive boundary layer which have heterogeneous distributions of dissolved oxygen during the day and become anoxic at night. Anaerobic metabolisms may be important on coral surfaces as corals have also been shown to be inhabited predominately by facultative anaerobes [12]. An interesting observation from the nutrient measurements is that islands with higher nitrate availability have lower ammonium concentrations whereas low nitrate islands have higher ammonium. Nitrate to ammonium ratios were 0.26, 0.29, and 0.22 on Malden, Jarvis, and Kiritimati compared to 3.23 and 1.47 on Flint and Kingman, respectively (data not shown). Therefore, the overrepresentation of DRNA may reflect the low abundances of ammonium at these sites.

Finally, genes for chemotaxis were more abundant at high nutrient sites (r=0.598, p = 0.052, Table 3.4). Mechanisms for chemotaxis (i.e., active motility) may enhance a cell's ability to acquire energy and nutrients, respond to quorum sensing cues, and may even contribute to increased gene transfer.

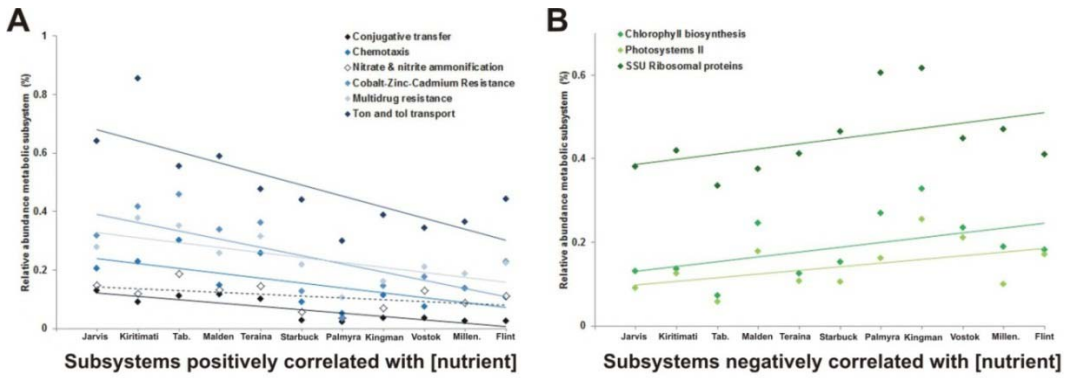

Figure 3.4. Metabolic pathways that correlate to nutrient concentrations across the Line Islands. Pathways are represented as level 3 subsystems annotated using the SEED database. Metabolic pathways are shown for those that both positively (A) and negatively (B) correlate with increasing nutrient concentrations. Tab., Tabuaeran. Millen., Millennium.

Low nutrient availability was correlated with metabolic pathways involved in photosynthesis, such as chlorophyll biosynthesis and photosystems I and II. (Figure 3.4 and Table 3.4). In this case, the abundance of autotrophic taxa influenced the genes present here. Metabolic pathways involved in protein metabolism were also overrepresented at oligotrophic sites, namely proteins that constitute the bacterial ribosomes small subunit ($r = 0.62$, $p = 0.042$, Table 3.4). Ribosomal proteins are generally encoded by single copy genes therefore, an overrepresentation of these genes at oligotrophic sites suggests that the associated microbes have smaller genomes (i.e., genome streamlining as described in *Pelagibacter ubique*) [1].

Table 3.4. Significance test for the linear correlations of metabolic pathways and phosphate concentrations.

Metabolic pathway	Pearson's coefficient r	p value
Conjugative transfer	0.863	**0.001**
Bacterial chemotaxis	0.598	0.052
Nitrate & nitrite ammonification	0.628	**0.038**
Cobalt-zinc-cadmium resistance	0.637	**0.035**
Multidrug resistance	0.617	**0.043**
Ton and tol transport	0.650	**0.03**
Chlorophyll biosynthesis	0.552	0.079
Photosystem II	0.534	0.091
Ribosome SSU bacterial	0.620	**0.042**

Community adaptation. Figure 3.5 depicts a conceptual model to describe the biology underlying the observed results of the metagenomic analysis. Metagenomic sequence reads match a subset of genes present in the genomes of sequenced bacterial

isolates in the database (represented as sequence similarities to genes A, D, F, G and J of Species 1, Figure 5). These five sequence similarities to “Species 1” are recorded in the taxonomic similarities output and determine the abundance of “Species 1” on a given reef. The function of these five genes is also recorded in the metabolic similarities output. These represent the “core genes” of “Species 1”. The genes depicted with black or blue hash marks represent metabolic genes not present in the genome of the “Species 1” isolate included in the database. These genes are included in the genome of the environmental isolate and represent the “dynamic genes” (e.g., pan, mobile, or auxiliary genes) that move laterally between microbial species and allow for adaptation to a particular niche (Hypothesis 1, Figure 3.5). Because these “dynamic genes” (depicted with black or blue hash marks) are either assigned to microbial taxa other than “Species 1” or assigned as unclassified, they illicit results where two environments can have similar microbial community structure but different community metabolism.

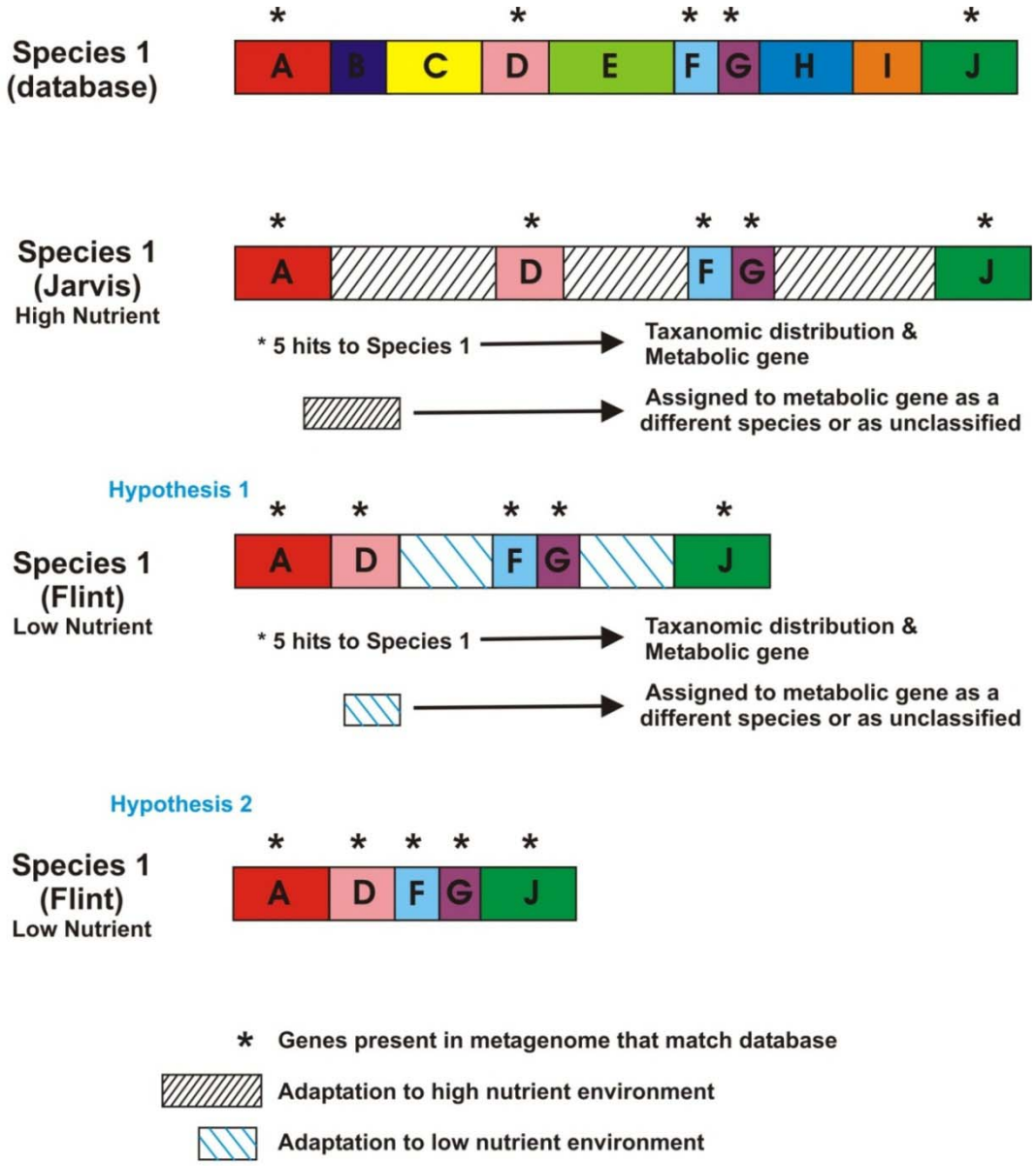

Figure 3.5. Conceptual model to explain differences in taxa abundance and community metabolism on LI reefs.

The results from this study show that community structure correlated best with benthic community composition. Previous research has shown that reef associated

bacterial communities are selected for based on the composition of the organic matter exuded by the benthic organisms [35], therefore we posit that on coral reefs, microbial community structure (microbial biogeography) is determined by the available carbon sources. The community metabolism is then influenced by the specific genes incorporated laterally in order to adapt to the environmental parameters on each island (e.g., nutrient availability). For reef associated microbial communities, higher nutrient availability selected for genes involved in increased membrane transport, conjugation, motility, nitrogen cycling, and for the efflux of metals and toxic compounds. Low nutrient sites selected for genes involved in autotrophy and protein metabolism. An alternative hypothesis is that high nutrient sites select for metabolic complexity in general, whereas oligotrophic sites selected for genomes more suited to steady state conditions (Hypothesis 2, Figure 3.5). Specifically, we observed that single copy genes which encode ribosomal proteins are overrepresented at oligotrophic sites suggesting that these genomes are smaller proportionally compared to the genomes at high nutrient sites.

This study is novel because it highlights the importance of dynamic genes in structuring the metabolic potential of a community of microbes. Other examples in nature demonstrate that microbial genomes adapt to environmental conditions through horizontal transfer of advantageous metabolisms. For instance, a genomic investigation of gut symbionts from a single honey bee colony found that isolated species sharing 99% identity in 16S rRNA genes differed in their ability to degrade pectin based on the presence of a pectate lyase gene [39]. Engel and colleagues

hypothesized that this strain variation amongst species reflected divergent niche adaptation within the gut as well as conferring fitness for the availability of different food sources.

In the ocean, cyanobacteria demonstrate different gene content and organization for phosphate acquisition based on the phosphate availability where the strains were isolated rather than on phylogeny. Here, horizontal gene transfer allowed for the acquisition of a few key genes rapidly changing the spectrum of nutrient sources available to the cell. Strains of Prochlorococcus, 99.9% similar in their 16S rRNA genes, differed in the types of phosphate genes present and in their location within the genome [7]. Conversely, some of the strains further diverged based on phylogeny were more similar in content and organization of phosphate genes because they occupied environments with similar nutrient regimes. The movement of nitrogen metabolism genes has also been demonstrated in *Prochlorococcus*, which typically only assimilates ammonium. *Prochlorococcus* strains inhabiting regions of nitrogen limitation were adapted to utilize nitrate and nitrite [8]. These genes for nitrate and nitrite assimilation in *Prochlorococcus* constitute “dynamic genes” acquired horizontally from other taxa (i.e., *Synechococcus*) in order to adapt to the environment.

Here, we demonstrated that the community metabolism of coral-associated microbes inhabiting coral surfaces and within the boundary layer reflects the local availability of nutrients. Greater nutrient availability was associated with more metabolic versatility whereby these microbes had an increased capacity for nutrient

transport, motility and chemotaxis, as well as transfer of genetic material via conjugation. One aspect of this research that is unclear is whether the metabolic genes observed to correlate with nutrient availability are moving horizontally through the community or whether strains of microbes with the advantageous genes are becoming more abundant (beta diversity). I hypothesized that horizontal gene transfer played a role, however further investigation is needed to determine the mechanism by which these patterns are observed.

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Author Contributions

The manuscript was written by LK. The metagenomic analyses were completed by LK. The multivariate statistical analyses were completed by GJW. Water samples for metagenomes and nutrient analysis were collected by KLB, CAC, EAD, AH, CEN, TM, SAS, ES and FR. The benthic characterizations were completed

by JES, GJW, and KLB. YWL and MH completed all of the library prep and sequencing reactions. RAE provided valuable computational support to LK. All of the authors offered helpful comments to the manuscript.

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Appendix

Supplementary Tables

Supplementary Table 3.1. Colinearity among predictor variables using Pearson's Coefficient, R. The correlations between distance from equator and nutrient concentrations are shown in bold.

	Hard coral	CCA	Other calcified algae	Soft coral	Macro-algae	Turf algae	Other benthic	Nitrate	Phos
Hard coral									
CCA	-0.536								
Other calcifying algae	-0.373	0.193							
Soft coral	-0.355	0.339	-0.089						
Macroalgae	-0.287	0.120	-0.151	0.001					
Turf algae	-0.533	-0.196	-0.297	0.213	0.051				
Other benthic	-0.548	0.196	0.294	-0.030	0.211	0.538			
Nitrate	-0.161	0.163	0.038	-0.025	0.105	0.077	-0.077		
Phosphate	-0.266	0.155	-0.175	-0.017	0.258	0.299	-0.088	**0.708**	
Distance from equator	0.487	-0.072	0.054	-0.071	-0.250	-0.531	0.316	**-0.641**	**-0.741**

Phos, Phosphate

Supplementary Table 3.2. Predictor variable categories used for canonical correspondence analysis and distance-based permutational multivariate multiple regression model (DISTLM). Benthic coverage for each functional group is shown as percent cover. Nutrient concentrations are calculated as micromoles per liter. Distance from equator is represented by the absolute value of the latitude in decimal degrees.

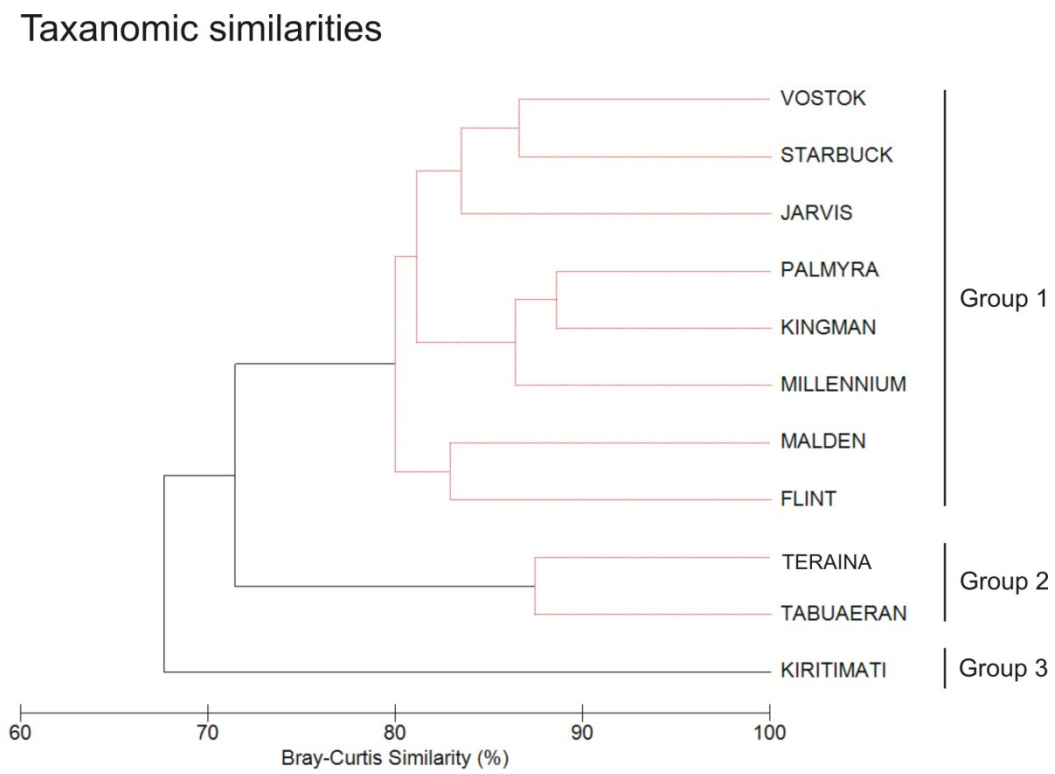
Sample name	Hard coral	CCA	Other calc. algae	Soft coral	Macro-algae	Turf	Other	NO_3^- (µM)	PO_4^{3} (µM)	Dist. from equator
Curacao 1	ND	ND	ND	ND	ND	ND	ND	0.70	0.10	12.122946
Curacao 2	ND	ND	ND	ND	ND	ND	ND	0.70	0.10	12.100948
Curacao 3	ND	ND	ND	ND	ND	ND	ND	0.70	0.10	12.369642
Flint 2	75.85	13.10	2.00	0.00	1.15	7.65	0.25	1.09	0.29	-11.41924
Flint 5	83.00	9.00	0.20	0.00	0.95	6.60	0.25	0.82	0.16	-11.43911
Flint 6	ND	ND	ND	ND	ND	ND	ND	0.79	0.15	-11.44423
Jarvis 4	46.30	27.20	0.70	0.00	7.90	17.70	0.30	4.65	0.38	-0.38188
Jarvis 9	57.90	9.30	0.00	0.00	3.40	29.20	0.20	4.54	0.43	-0.36537
Jarvis North	10.70	31.90	1.00	0.00	4.90	50.30	1.30	4.50	0.44	-0.36902
Jarvis Tent	57.65	12.00	0.15	0.35	6.90	21.65	0.95	3.28	0.39	-0.369017
Kingman 2	14.55	51.20	12.70	0.40	0.95	18.25	1.95	1.44	0.25	6.387
Kiritimati Oil	2.21	0.00	6.68	0.00	4.00	82.47	4.63	2.26	0.24	1.99095
Kiritimati Tent	23.36	2.50	7.77	1.64	0.14	58.86	5.73	2.33	0.29	2.0085833
Malden 25	73.63	5.37	1.84	0.00	1.79	15.95	1.42	3.90	0.26	-4.03326
Malden 5	86.67	4.56	0.00	0.00	0.22	8.11	0.44	2.82	0.25	-3.99531
Millennium 12	65.30	11.00	12.30	0.00	1.10	10.10	0.20	2.28	0.22	-9.90774
Millennium 9	69.30	6.20	7.70	0.00	0.30	15.70	0.80	2.10	0.19	-9.91672
Moorea	ND	ND	ND	ND	ND	ND	ND	0.41	0.13	-17.48642
Palmyra 1	45.70	16.30	5.60	2.00	1.40	23.90	0.60	0.52	0.19	5.86646
Starbuck 13	25.55	12.35	57.95	0.00	0.10	0.20	3.85	2.87	0.25	-5.66441
Starbuck 7	21.68	49.42	21.84	0.00	1.21	4.47	1.21	4.83	0.25	-5.6222
Tabuaeran 10	22.08	30.08	34.62	0.00	8.69	3.92	0.46	2.78	0.30	3.82595
Tabuaeran 2	39.23	20.18	16.32	0.00	2.41	17.86	4.00	1.60	0.19	3.84085
Teraina 2	20.96	21.96	0.00	0.76	32.12	20.44	3.76	2.24	0.28	4.70242
Teraina Tent	8.64	44.93	6.64	6.36	2.36	30.79	0.29	1.92	0.28	4.6867167
Vostok 10	81.40	14.40	0.35	0.00	0.15	3.70	0.00	1.75	0.16	-10.05835
Average	**44.36**	**18.71**	**9.35**	**0.55**	**3.91**	**21.33**	**1.55**	**2.22**	**0.24**	**-**

calc., calcified. Other, other benthic organisms, NO_3^-, nitrate + nitrite, PO_4^{3}, phosphate. Dist., distance.

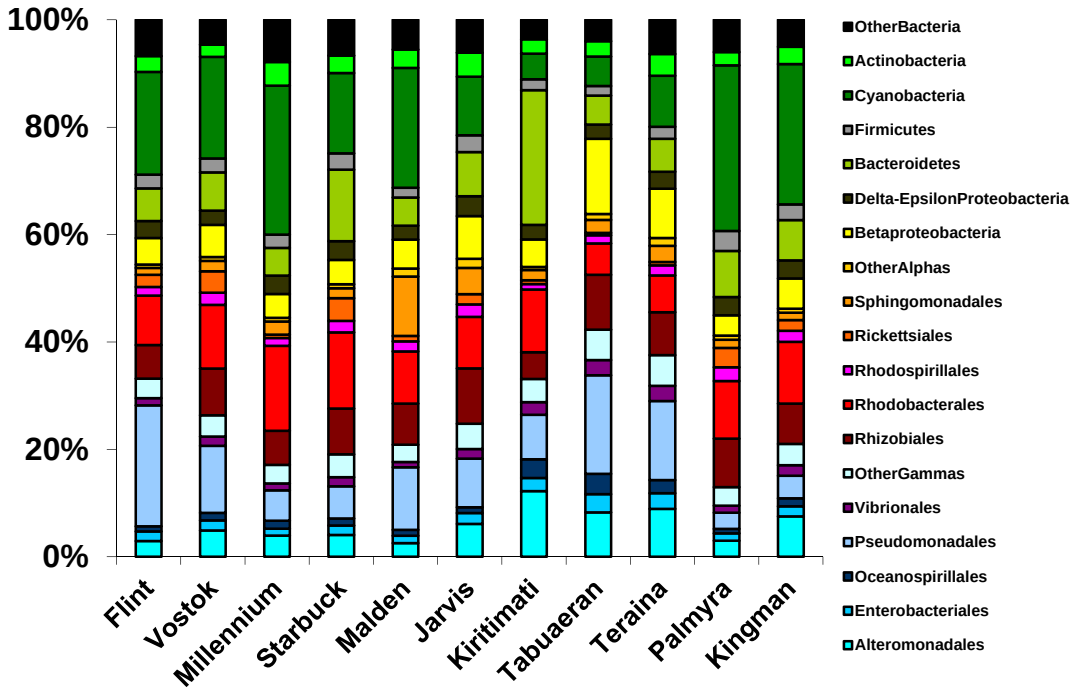
Supplementary Table 3.3. Summary of the benthic taxa that were classified into seven functional groups. Crustose coralline algae (CCA) and turf algae were identified as functional groups only.

Hard coral
Acropora, Astreopora, Cyphastrea, Cycloseris, Echinophyllia, Favia, Favities, Fungia, Gardineroseris, Halomitra, Herpolitha, Hydnophora, Leptastrea, Leptoseris, Lobophyllia, Montastrea, Montipora, Pavona, Platygyra, Pocillopora, Porites, Psammocora, Sandolitha, Scapophyllia, Sytlophora, Tubastrea, Turbinaria
Other calcified macroalgae
Galaxaura, Halimeda, Neomeris, Peyssonnelia
Soft coral
Dendronephtya, Leather coral*, Pachyclavularia, Sarcophyton, Stereonephthya*
Macroalgae (fleshy)
Avrainvillea, Brown crust*, Caulerpa, Cladophora, Dictyosphaeria, Dictyota, Hypnea, Lobophora, Valonia*
Other benthic organisms
Asteroidea, Cyanobacteria, Echinothrix, Heteractis, Heterocentrotus, Holothurian, Hydroid*, Millepora, Rhodactis,* Sand, Sponge*, Stylaster, Tridacna,* Tunicate, Zoanthid

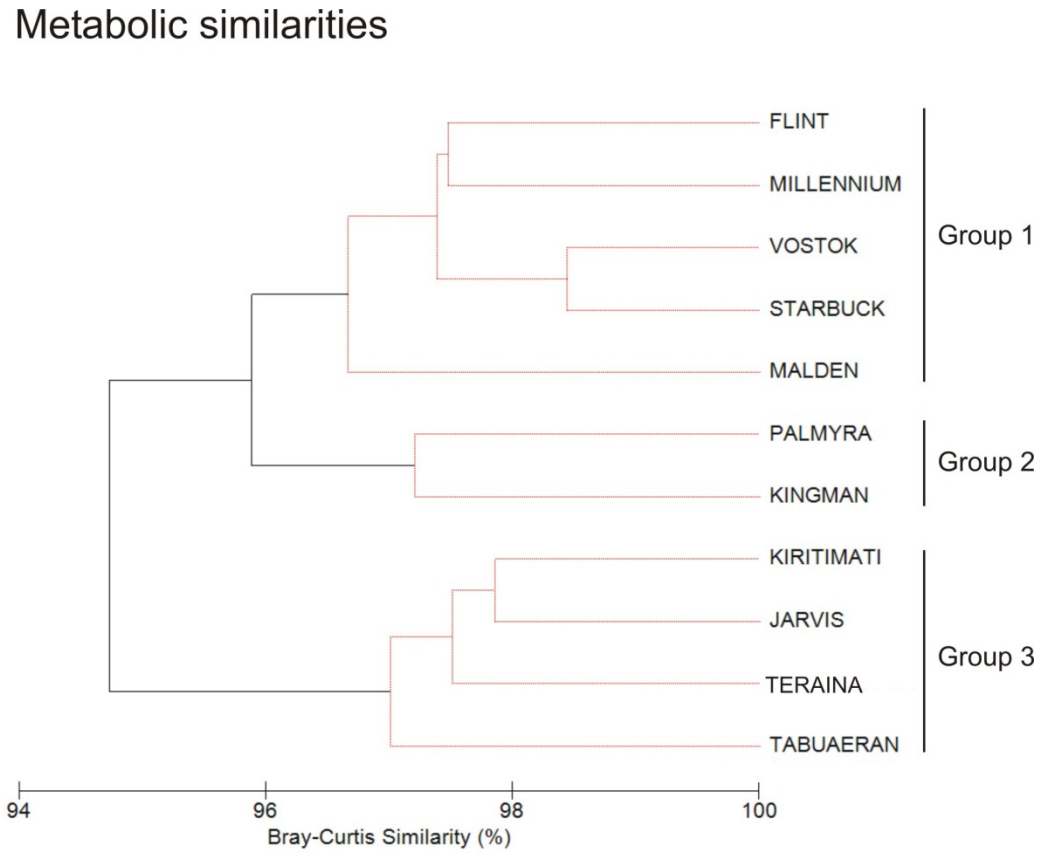
Supplementary Figures



Supplementary Figure 3.1. Taxonomic groupings by SIMPROF.



Supplementary Figure 3.2. Bar chart depicting the taxonomic distribution for the LI bacterial communities.



Supplementary Figure 3.3. Community metabolism groupings by SIMPROF

CHAPTER 4: Black reefs: Iron induced phase-shifts on coral reefs

The Line Islands (LI) are calcium carbonate coral reef platforms located in iron-poor regions of the central Pacific. Natural terrestrial run-off of iron is non-existent and aerial deposition is extremely low. However, a number of ship groundings have occurred on these atolls. The reefs surrounding the shipwreck debris are characterized by high benthic cover of turf algae, macroalgae, cyanobacterial mats, and corallimorphs, as well as particulate-laden, cloudy water. These sites also have very low coral and crustose coralline algal (CCA) cover and are call *black reefs* because of the dark colored benthic community and reduced clarity of the overlying water column. Here we use a combination of benthic surveys, chemistry, metagenomics, and microcosms to investigate if and how shipwrecks initiate and maintain black reefs. Comparative surveys show that live coral cover was reduced from 40-60% to <10% on black reefs on Millennium, Tabuaeran, and Kingman. These three sites are relatively large (>0.75 km^2). The phase-shift occurs rapidly; the Kingman black reef formed within 3 years of the ship grounding. Iron concentrations in algae tissue from the Millennium black reef site were 6-times higher than in algae collected from reference sites. Metagenomic sequencing of the Millennium Atoll black reef-associated microbial community was enriched in iron-associated virulence genes and known pathogens. Microcosm experiments showed that corals were killed by black reef rubble via microbial activity. Together these results demonstrate that

shipwrecks and their associated iron pose significant threats to coral reefs in iron-limited regions.

Introduction

The Line Islands (LI) located in the central Pacific harbor several nearly pristine coral reefs [1]. The islands' emergent terrestrial substrates are calcium carbonate reef sands and rubble, devoid of emergent basaltic crust that would release iron into the surrounding waters [2]. Most of the islands are uninhabited and are isolated from point sources of pollution. Shipwrecks are one exception and have been documented on Kingman, Millennium, Palmyra, Starbuck, and Tabuaeran. There are also shipwrecks on similar carbonate atolls throughout the Pacific, including McKean, Canton, Enderbury, Phoenix, and Nikumaroro of the Phoenix Islands, and on Rose Atoll of American Samoa [3-5]. In the Line Islands, the areas surrounding the shipwrecks become *black reefs*, characterized by high prevalence of fleshy algae—both turf and macroalgae, cyanobacterial mats and corallimorphs and a dramatic loss of corals and crustose coralline algae (CCA) [4, 5].

Similar phase-shifts in response to shipwrecks where coral and CCA dominated communities change to communities dominated by opportunist benthic organisms have been documented on other coral reefs (the term *phase-shift* refers to the observation described by Done [6], where environmental conditions favor algal overgrowth of previously coral-dominated ecosystems). On Rose Atoll, the percent cover of turf /cyanobacterial assemblages was an order of magnitude higher (>40%) on reefs surrounding the wreck compared to reference sites on the same island. This algal community persisted despite the presence of significantly higher abundances of herbivorous fishes at impacted sites [4]. The corallimorph *Rhodactis howesii* has

spread over the previously coral dominated reef terrace on Palmyra Atoll in response to shipwreck debris [5]. High densities of the rapidly proliferating *R. howesii* extended >2 km from the shipwreck, though the abundances progressively declined with distance. Although this work is most pertinent to remote Pacific islands with low iron inputs, coral reefs located on continental shelves may also be susceptible. A bulk carrier which ran aground on the windward side of Myrmidon Reef, GBR, initiated a phase-shift where the red macroalgae *Asparogopsis taxiformis* dominated the benthos and live coral cover was diminished to <1% [7]. A different circumstance where coral reefs were subjected to iron fertilizations comes from the aftermath of extensive wildfires in Indonesia. Iron enrichment resulting from ash deposited onto the Mentawai reefs located offshore southwest of Sumatra, Indonesia, led to an explosive dinoflagellate bloom that suffocated and killed nearly 100% of corals [8]. In summary, marine ecosystems occurring in regions with low iron availability can be substantially altered by iron enrichment.

Iron concentrations are extremely limited in the central Pacific ocean [9] with concentrations ranging from 0.2 –1 nM [10]. Few studies have measured the iron available to the benthic organisms on coral atolls. However, sediments on the reefs consist mostly of calcium carbonate generated *in situ* through the erosion of calcifying organisms and as such unlikely to contain much iron [11]. We hypothesize that: 1) iron limits primary production by algae and cyanobacteria on central Pacific coral atolls where there are no emergent basaltic rocks, 2) on these atolls shipwreck-associated iron releases these primary producers from bottom-up controls, and 3) the

resulting communities of fleshy algae and cyanobacteria either directly compete with the coral or they promote microbes that kill the coral [12, 13]. In this study we document three black reefs in the LI, measure iron concentrations directly from coral reef algae, investigate the effects of iron enrichment on corals, algae, and their associated microbes, and characterize the microbial community associated with a black reef using metagenomics. Together our results support the hypothesis that shipwreck iron is capable of inducing the collapse of reef systems in iron-depleted oceans such as the LI.

Results and Discussion

Five of the LI have shipwrecks on them resulting in black reefs that are characterized by low coral and CCA cover. As shown in Figure 1, the benthos at these sites appears very dark red or black with increased abundances of turf algae, macroalgae, cyanobacterial mats, and/or corallimorphs. The surrounding water is cloudy with elevated concentrations of particulate organic matter. We surveyed the three largest black reefs and describe their natural history below.

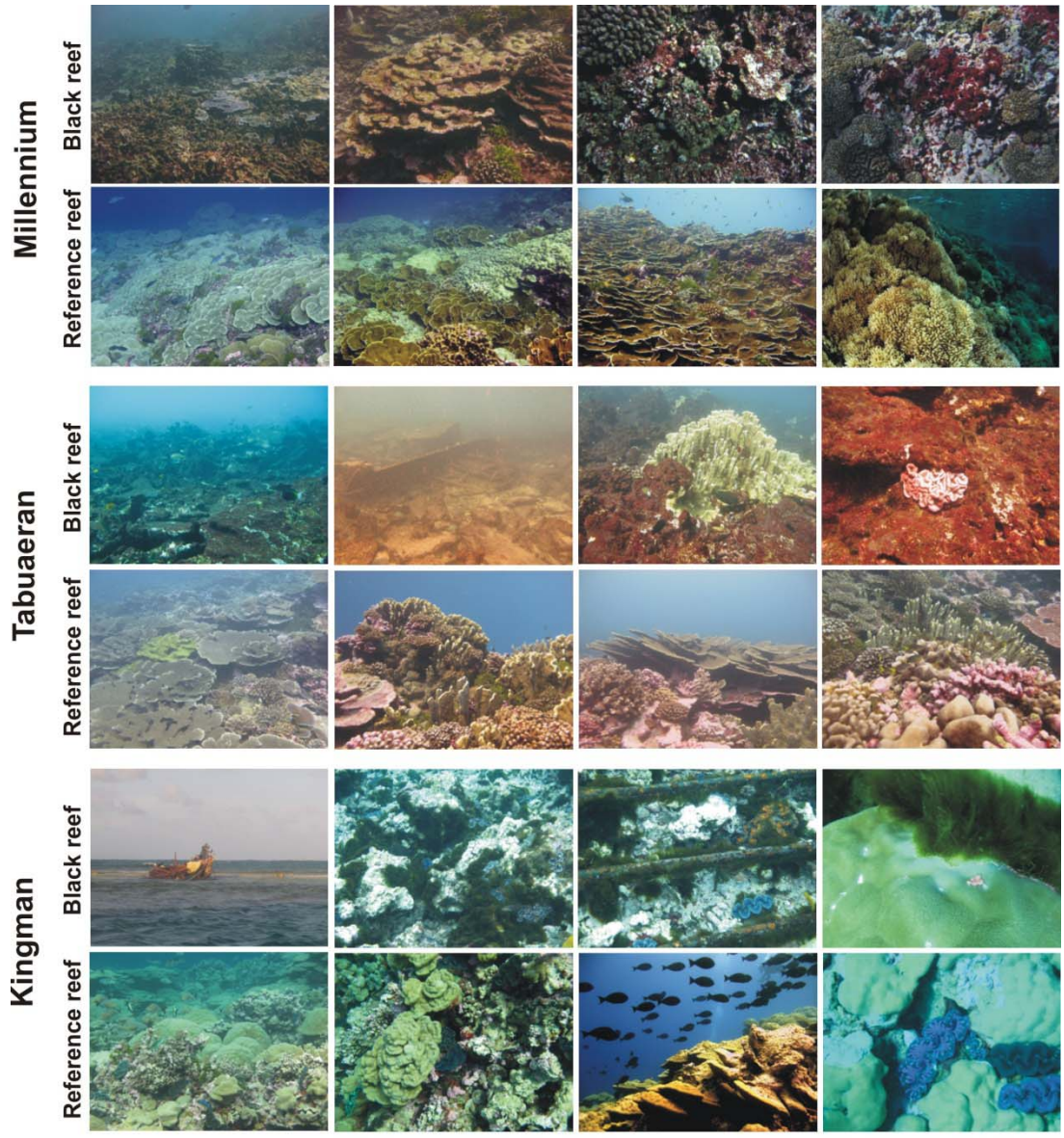


Figure 4.1. Representative photographs of benthic habitats from black reefs and reference reefs on Millennium (rows 1, 2), Tabuaeran (rows 3, 4), Kingman (rows 5, 6).

Natural History of the Millennium Atoll Black Reef: Millennium, formerly Caroline Island, is the most southern atoll of the Line Islands (Lat: -9.955, Long: -150.210). The reefs on Millennium were characterized by high fish abundances [14], high abundances of stony corals and CCA, and low abundances of benthic algae (Figure 4.1). The 1993 shipwreck occurred when an 85 ft steel tug ran aground while towing a sailing vessel out of a narrow reef passage. Figure 4.2A compares the benthic community composition from the black reef with other sites around the atoll. The Millennium black reef extended for 0.75 square kilometers down current of the shipwreck debris field. Live coral cover was the most abundant benthic functional group on reference sites around Millennium (Figure 4.2A, mean value 65.9%, N = 10, SE ± 4.5) compared to 16.7% (N = 10, SE ± 4.3) on the black reef. The algal assemblages present on the Millennium black reef are predominately turf algae and cyanobacterial mats.

Natural History of the Tabuaeran Black Reef: Tabuaeran Atoll, located north of the equator (Lat: 3.880, Long: -159.320), historically supported guano mining and copra farming, and is currently inhabited by ~ 2,500 people. One of ships used in the copra trade ran aground just north of the lagoon channel on the leeward side of the atoll. The black reef associated with this wreck now covers approximately 10% of the surrounding reef (i.e., 1/10 of the circumference of Tabuaeran). The spread of the black reef appears to be inhibited only by the two natural barriers created by the strong lagoonal current in the south and the high energy, sand-bottom surf zone in the North (surveyed by Rohwer and Vermeij in 2010). The black reef consisted predominately of

dead coral and rubble covered with thick cyanobacterial mats and turf algae (Figure 4.1). The reefs at the shipwreck site were investigated in August 2005 and November 2010. In 2005, live coral cover was < 5% compared with 40.2 % ± 14.8 and 28.9% ± 5.1 on leeward reef sites north and south of the wreck, respectively (Figure 4.2B). In 2010, there was no coral recovery in the sites surveyed in 2005 and the black reef had extended to the lagoon entrance. Though we do not know the exact date of this shipwreck, it is at least 40 years old (Maragos, J.E., personal communication), showing that these wrecks cause persistent reef degradation and represent a long-term threat to LI reefs.

Natural History of the Kingman Black Reef: Kingman, an atoll located in the northern-most region of the LI (Lat: 6.400, Long: -162.380), was a National Wildlife Refuge until 2009 when it became a US Marine National Monument. This protected pristine ecosystem exhibits an inverted biomass pyramid (85% of the total in top predators), high coral recruitment and coral cover, and the second highest coral diversity in the central Pacific [3, 15]. The shipwreck here is a teak-hulled fishing vessel filled with iron-rich compressors, engines, and unidentifiable machinery. When first observed in 2007, the hull was located on the fore-reef side of the northeast islet. By November 2010, the wreck had been pushed across the islet and debris was spread across both the fore- and back reef.

The reef surrounding the Kingman shipwreck has been overgrown by the filamentous green algae *Derbesia tenuissima* (Tsuda, R. T., personal communication; Figure 4.1). At the wreck site, *D. tenuissima* covered up to 80% of the benthos while

coral plus CCA cover declined to <10% (compared to <15% turf plus macroalgae and ~50% coral plus CCA in 2007, Figure 4.2C). *D. tenuissima* overgrowth extended 1.5 km along the reef horizontally and algal overgrowth was observed down the reef slope to ~35 m (Rohwer and Williams, personal observation 2010). Since *D. tenuissima* was not previously observed in this region, it may be invasive and/or have been introduced by the ship.

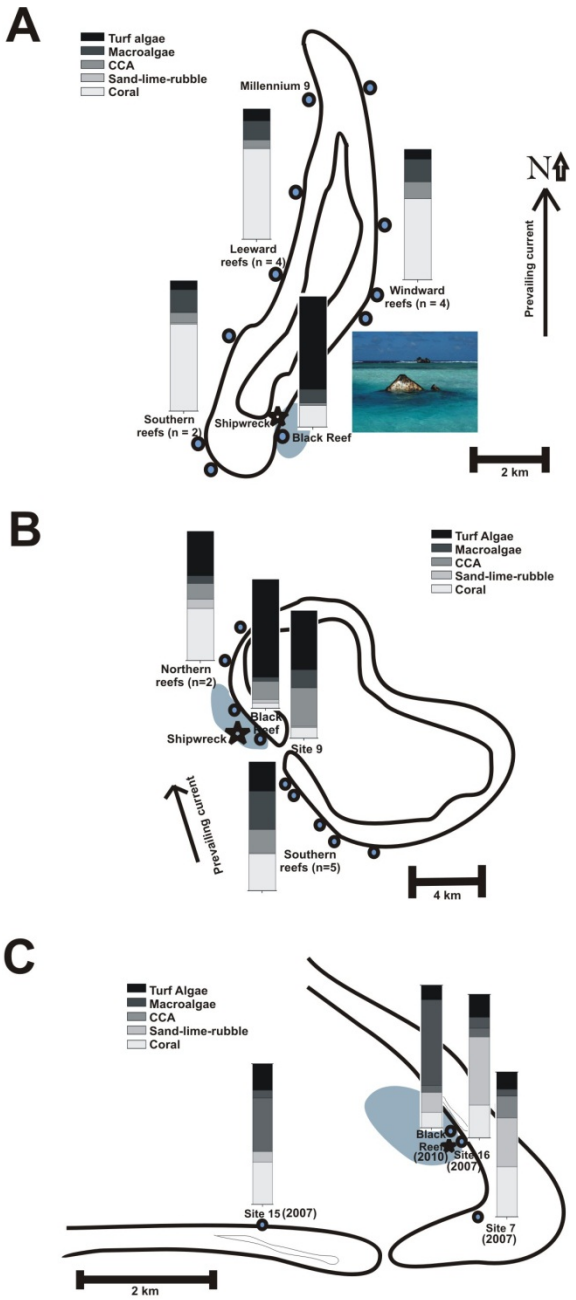


Figure 4.2. The benthic community composition present on Millennium Atoll (A), Tabuaeran Atoll (B), and backreef sites on Kingman Reef (C). On Kingman, Sites 7, 15 and 16 were measured in August 2007 prior to the influence of shipwreck debris. The black reef site was measured in October 2010. The area of reef estimated to be impacted by shipwreck debris is indicated with light blue shading. The bar graphs represent the percent cover of coral, crustose coralline algae (CCA), macroalgae, and turf algae (0.72 m^2 quadrats per site, n = 20). The black arrows indicate the direction of the prevailing current.

Other examples of black reefs in the central Pacific: Similar black reefs have been observed on Canton, Enderbury, McKean, Nikumaroro, and Phoenix of the Phoenix Islands (Supplementary Fig. 1). The Millennium, Tabuaeran, and Kingman black reefs cover relatively large areas; however, we have also observed small versions of the same phenomenon around lesser localized iron sources (e.g., lost anchors, steel piping) on Enderbury (Supplementary Fig. 2A) and Jarvis (Supplementary Fig. 2B & C).

Summary of LI black reefs: The natural history of these black reefs shows that they: 1) are always congruent with shipwrecks or a point source of iron; 2) can develop quickly after a shipwreck (<3 years in the case of Kingman); 3) persist for decades (e.g., Tabuaeran and Millennium); 4) most consistently and abundantly include a source of iron (e.g., not copper paint, petroleum products, etc); and 5) have similar biotic composition (turf algae, macroalgae, cyanobacterial mats, and corallimorphs), with variation in the relative abundances of these groups. This variation may represent stages of succession or differences in the biogeography of specific taxa.

The LI occur in a high-nutrient, low-chlorophyll (HNLC) region of the ocean. Paradoxically, HNLC regions have low primary production despite higher concentrations of phosphate and nitrogen. Martin et al. [16] resolved this apparent paradox by showing that HNLC waters are iron limited; addition of iron leads to a rapid increase in primary production. Figure 4.3A depicts the estimated iron concentrations for the world's oceans based on iron deposition. Given this regional

background and the observation that iron points sources are associated with all black reef sites, we hypothesized that iron supplementation was supporting the large-scale algal overgrowth of corals on black reefs.

Comparison of iron concentrations in mixed algal assemblages. To investigate whether there was more bioavailable iron on the black reef, the incorporation of elemental iron into algal tissue was measured using inductively coupled plasma (ICP-OS). Iron concentrations measured in mixed algal assemblages from the Millennium black reef were 6-times higher than in assemblages from reference sites (Figure 4.3B and Supplementary Table 4.1; median values 633 μmol Fe : mol C at the black reef versus 105 μmol Fe : mol C at Millennium reference sites and 83 μmol Fe : mol C at other LI control sites). The iron concentrations for the black reef were significantly different from reference sites on Millennium (Mann-Whitney U-test, two-tailed, n = 10, P value = 0.009) and from reference sites on other LI reefs (n = 11, P value = 0.018).

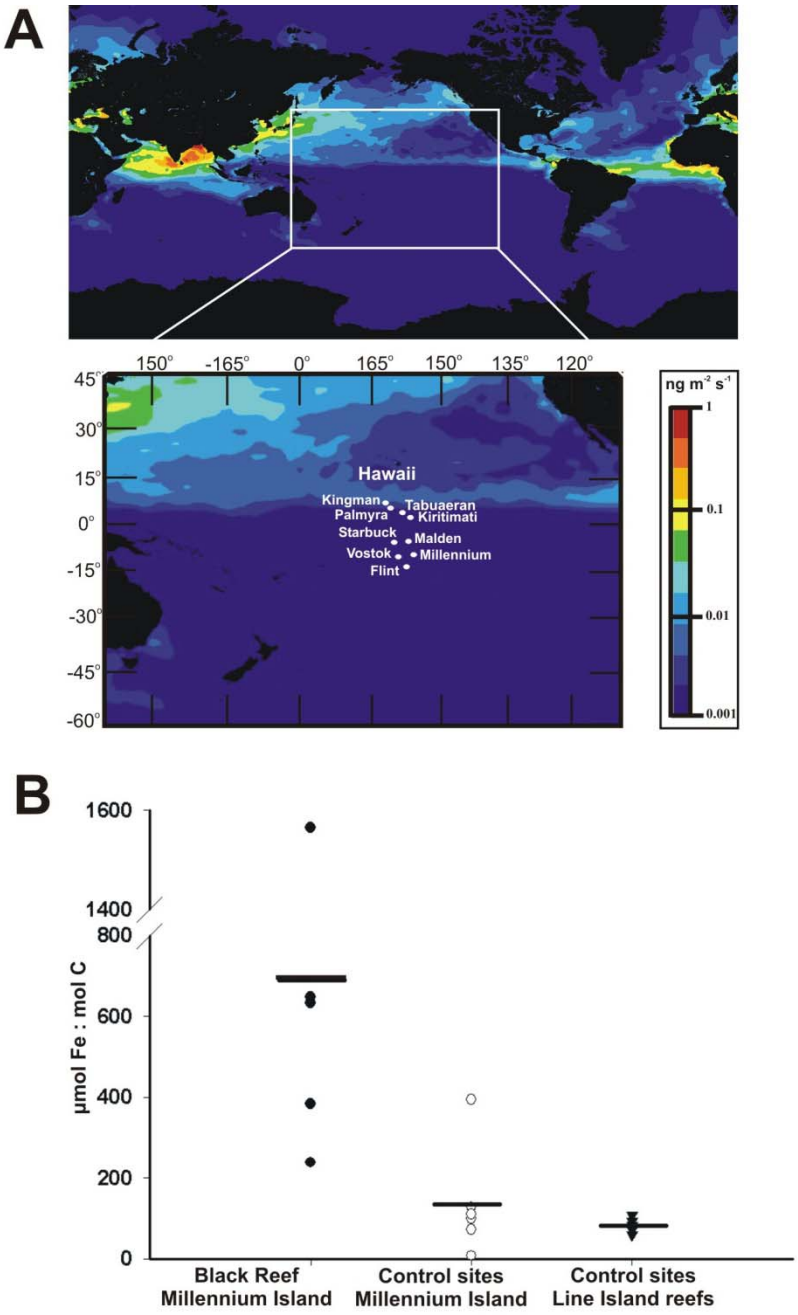


Figure 4.3. Regional and local iron concentrations. (A) Iron concentrations across the world with an emphasis on the central Pacific (data for oceanic iron concentrations were taken from Behrenfeld et al. 2009). The colored bar estimates iron deposition (ng m^{-2} s^{-1}). (B) The elemental stoichiometry of iron incorporated into the tissues of benthic organisms from the black reef, control sites on Millennium, and from other Line Island reefs. Iron and carbon concentrations were measured using ICP-OS and EA, respectively. The horizontal line represents the mean of each dataset.

For these comparisons, mixed algal species (composed mainly of red algae and CCA) were isolated from coral rubble (Supplementary Table 1). Consequently, the proportion of different algal species present in each sample could not be quantified. Although some deviations in iron concentration may be attributable to differences in algal species composition, the large differences observed in this study likely reflect environmental iron availability. The iron concentrations of mixed algae collected from LI reference sites were comparable to those found in macro- and turf algae from Davies Reef, GBR (Supplementary Table 1; [11]. Although the iron concentrations in filamentous cyanobacteria on Davies Reef were 2- to 4-fold higher than in species of red and green macroalgae, the iron concentrations of mixed algal assemblages from the Millennium black reef were 3-fold higher than the Davies reef cyanobacteria and nearly an order of magnitude higher than the average Davies Reef macroalgae. In addition to iron, 13 other elements (magnesium, zinc, manganese, selenium, nickel, lead, chromium, cadmium, copper, mercury, arsenic, sulfur, and strontium) were measured in samples collected from the black reef using ICP. None of these metals were enriched at the shipwreck sites compared to reference sites (data not shown).

The increased iron concentrations in algal assemblages adjacent to the shipwreck debris indicates an increase in bioavailable iron. While the form of iron released from the corroding ship (e.g., $Fe(OH)_3$) may not be immediately available for uptake by algae, ferric iron can be solubilized through the complexation of high affinity organic ligands which substantially increases its bioavailability [17, 18]. Although the complexites of iron biogeochemistry are largely unknown [19], there is

compelling evidence that the insoluble iron pool is rapidly complexed, thus increasing its bioavailability to microbes and eukaryotic algae [20-22]. The specific mechanism whereby the iron associated with the shipwreck debris is becoming available to the benthic algae has not been determined; nevertheless it is evident that iron is being recycled in marine environments and is accumulating in the algae around the Millennium black reef.

The effects of iron enrichment on coral health. The effects of iron enrichment on coral and coral/algal communities were investigated using *Pocillopora meandrina* corals collected from a healthy reference site on Tabuaeran maintained in mesocosms. Corals were incubated for five days in the presence or absence of ferric chloride ($FeCl_3$) and in the presence or absence of algal-covered rubble collected from the black reef on Tabuaeran (Fig. 4). Corals incubated with both iron and rubble communities showed the greatest mortality (Figure 4A & B, 50% dead, 20% dying), followed by the coral plus rubble treatment (20% dead, 30% dying). Enhanced microbial activity was previously linked to mortality of corals in the presence of macroalgae [12]. To determine whether the coral mortality observed here was mediated by microbes, the same treatments were conducted with antibiotics added. The addition of ampicillin significantly reduced coral death in the presence of iron and rubble (10% dead, 10% dying, Figure 4.4B, Mann-Whitney U-test, two-tailed, n = 20, P value = 0.007).

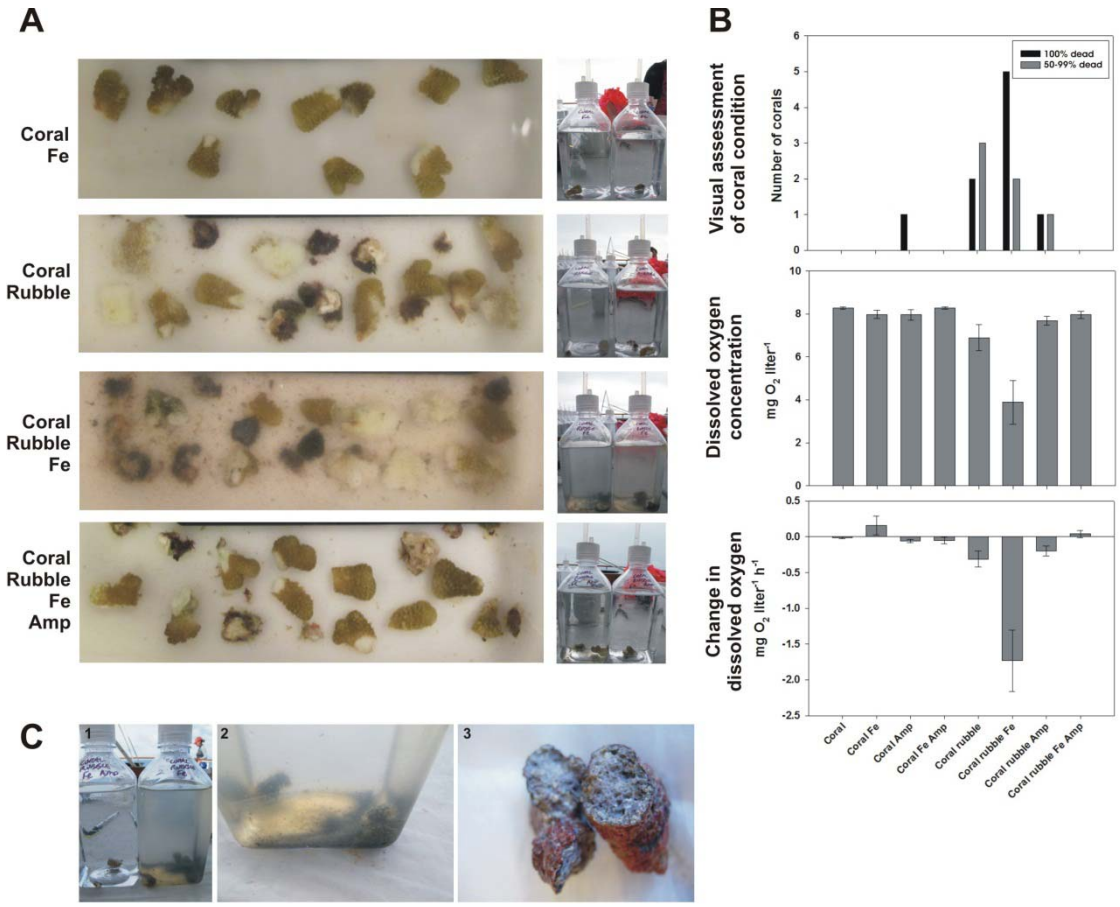


Figure 4.4. Mesocosm experiments measuring the response of corals and algal-covered rubble to iron enrichment. (A) *Pocillopora meandrina* corals were treated with 1 µM ferric iron ($FeCl_3$) with and without the presence of black reef algae. The same treatments were conducted plus 50 µg ml^{-1} amplicillin. Coral fragments and mesocosms were photo documented after five days (4 of 8 treatments shown, N = 10). (B) Coral condition was visually assessed (top panel). The concentration of dissolved oxygen (mg O_2 l^{-1}) in the aquarium water was measured at the completion of the experiment (middle panel). The rate of change in dissolved oxygen (mg O_2 l^{-1} hr^{-1}) was also measured (bottom panel). Fe, ferric iron. Amp, ampicillin. (C) Photographs of iron enriched mesocosms depicting water turbidity (1) and the formation of black particulate material characteristic of the black reef (2). Cross-section of algal-covered rubble collected from the Millennium black reef (3)

The water in the iron-enriched mesocosms turned cloudy, suggesting possible elevated growth rates of phytoplankton and heterotrophic microbes (Bacteria and Archaea) (Figure 4.4C). Iron availability is known to stimulate heterotrophic microbial production indirectly through the release of phytoplankton-derived dissolved organic matter [23]. Therefore, dissolved oxygen (DO) consumption was used to estimate respiration by heterotrophic microbes in these mesocosms. After five days incubation with added iron plus rubble, the concentration of DO was reduced from 7.9 ± 0.2 mg O_2 liter^{-1} in controls to 3.9 ± 1.1 mg O_2 liter^{-1} (Figure 4.4B), while DO consumption increased from 0.06 ± 0.05 mg O_2 liter^{-1} hour^{-1} to 1.7 ± 0.2 mg O_2 liter^{-1} hour^{-1}.

Metagenomic analysis of the microbial community associated with a black reef. Metagenomic sequencing was used to compare the microbial community associated with the Millennium black reef with that on near-pristine reefs in the region, including a healthy reference site on Millennium. In this study, six libraries with a total of 237, 282 sequences were obtained from six sites at four southern LI atolls: Millennium black reef; reference sites on Millennium, Flint (2), Starbuck, and Vostok (Table 4.1). Data for four atolls in the northern LI [24] were also included in the analysis. Metabolic profiles were created for each site based on the relative abundances of sequences similar to known sequences assigned to 25 SEED metabolic subsystems. The ten subsystem profiles were compared simultaneously by non-metric multidimensional scaling (nMDS). The Millennium black reef site grouped with Kiritimati, Kingman and Palmyra, and showed closest similarity to the degraded Kiritimati reef, on a scatter-plot of the first two nMDS dimensions (Figure 4.5). A

similarity profile test (SIMPROF) determined three significant groupings within the data set, mirroring the patterns visualized by the nMDS plot (Supplementary Fig. 3). The metabolic profile of the microbial community from the Millennium black reef differed significantly from the Millennium control site. Pathways involved in heterotrophic metabolism were 10-fold higher on the black reef (data not shown). Although sequence similarities to genes do not represent levels of gene expression, metagenomes have been shown to be strong predictors of the biogeochemical conditions driving the microbial community [25].

Table 4.1. Total number of sequences from each metagenomic library

Library Name	Average Sequence Length	Total No. Sequences	Total Library Size (bp)	Total No. Bacterial Similarities	Total No. Subsystem Similarities
Millen. black reef	234.7	46782	10979616	14053	9826
		Reference Sites			
Millen. 9	250.2	7340	1836674	980	782
Flint 2	240.0	21441	5145171	1620	3170
Flint 5	243.4	32832	7990372	1731	578
Starbuck 7	240.3	39335	9453923	4989	5865
Vostok 10	233.7	89552	20924215	14685	4308
Total	**240.4**	**237282**	**56329971**	**38058**	**24529**

No., number. bp, base paris. Millen., Millennium

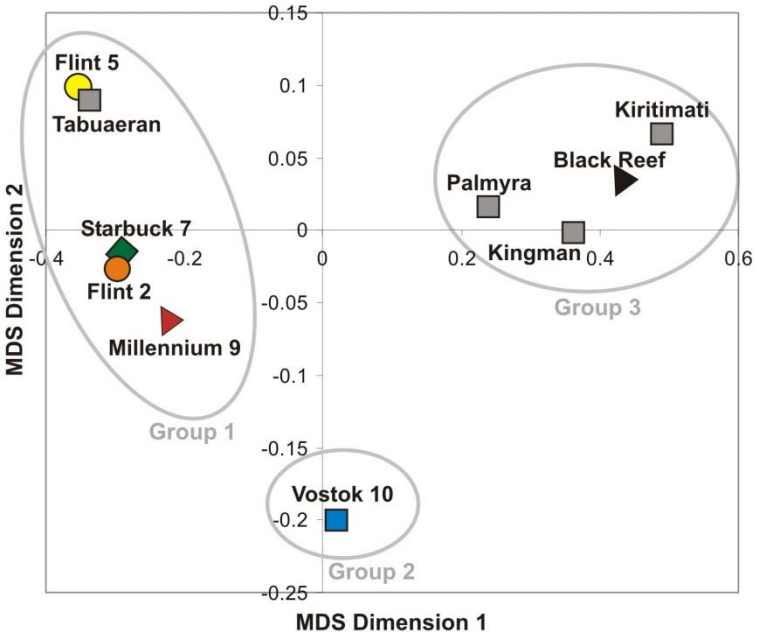


Figure 4.5. Metabolic profile for the microbial community inhabiting the black reef compared to nine other coral reefs. The scatter-plot depicts the first two dimensions of the non-metric multidimensional scaling (nMDS) analysis. The ten microbial metagenomes used for this analysis include six southern and four northern LI coral reefs [24]. Metagenomic libraries collected from northern LI reefs are depicted with gray squares. Metagenomic libraries collected from the same atoll are depicted with the same symbol (e.g., circles for Flint and triangles for Millennium). The three grouping shown were determined by a similarity profile test (P = <0.05).

Phylogenetic characterization of the microbial community supported the metabolic profiles. The black reef had proportionally more heterotrophic microbes than the reference sites on Millennium, Flint (two sites), and Starbuck (Supplementary Fig.4.4). Additionally, the microbial abundances were 3-times higher on the Millennium black reef ($20.5 \times 10^5 \pm 4.0 \times 10^5$ cells ml^{-1}; n = 3) compared to all other Millennium reef sites ($7.4\times 10^5 \pm 0.7 \times 10^5$ cells ml^{-1}; n = 23). The differences in microbial abundances were statistically significant (Mann-Whitney U-test, two-tailed, P value = 0.016). These changes in the microbial community are similar to previous observations by Dinsdale and colleagues where the degraded, algae-cover reefs of Kiritimati Atoll were dominated by heterotrophic, pathogen-like microbes [24]. One implication of this community shift is an increase in potential opportunistic coral pathogens [26, 27].

Virulence genes are overrepresented in black reef associated microbial community. Greater abundances of pathogen-like microbes and virulence factor genes were observed on degraded reefs [24], as well as in stressed corals [28]. To determine if virulence genes increased on black reefs, the six metagenomic libraries were compared to the virulence factor database (VFDB) [29]. The black reef sequences included 1068 significant similarities (Table 4.2; BLASTx; E-value < 0.0001), which when normalized for library size represented a 2- to 10-fold greater abundance of virulence genes than the other sites. More telling, virulence genes related to iron comprised 12% of the total virulence genes (126) on the black reef compared to 0 – 5% for the other sites (Table 4.2). These results suggest that iron enrichment may

select for a subset of potentially virulent microbes that can better scavenge and import iron.

Table 4.2. Abundances of virulence factor genes from microbes associated with Southern LI reefs

Site	Hits to virulence database	Normalized to total bp ($\times 10^5$)	Iron related virulence genes
Millen. black reef	1068	9.7	126
Millen. 9	51	2.8	3
Flint 2	230	4.5	4
Flint 5	18	0.2	0
Starbuck 7	341	3.6	15
Vostok 10	244	1.2	3

bp, base pairs. Millen., Millennium

Implications of coral to algal phase shifts for the associated microbial community. These changes in the Millennium black reef microbial community suggest an influence by the benthic algal overgrowth, in addition to the iron enrichment. Here the proposed mechanism is the stimulation of heterotrophic microbes by dissolved organic carbon (DOC) released by the benthic algae, which in turn leads to asphyxiation of the coral [12]. Turf algae on coral reefs can exude DOC at rates of 12.2 ±2.1 mg C m^{-2} hr^{-1} [30], whereas corals are net consumers of DOC [31]. This suggests that the composition of the benthic community can influence the availability of organic matter, thereby providing a possible mechanism for the observed shifts in the microbial community associated with the black reef. The increase in potential pathogens observed on the black reef might be contributing to coral death and/or inhibiting coral recovery [13], thus further increasing the available DOC. Furthermore, the mesocosm experiments demonstrated that black reef rubble algae were lethal to

corals even without iron enrichment, thus predicting long term consequences of the phase shift after removal of the iron.

Caveats: Due to the remote nature of these sites, we do not have complete datasets from all of the black reefs. Future studies need to include ICP-OS analysis of the algae and metagenomic analysis of the microbial communities from the other sites whose black reefs are of different ages. Current observations indicate a benthic succession following a shipwreck perturbation. Corallimorphs are common around the 1991 Palmyra shipwreck, but only present on the leading edges of the younger black reefs on Kingman and Millennium, and absent from the >40 year-old Tabuaeran black reef.

One drawback of our experimental design was that we could not compare iron concentrations of the same algal species on black reefs and reference reefs. Neither the dominant turf-cyanobacterial assemblages on Millennium/Tabuaeran or the green alga prevalent on Kingman were visibly present at reference sites. To address this, algae should be transplanted from reference sites to black reefs and their iron concentrations measured subsequently. Dissolved iron concentrations in the water column surrounding the wrecks should also be measured.

While sunken ships on some tropical reef flats may promote coral growth by providing a substratum for settlement, these artificial reefs are successful in regions, such as Hawaii or the Caribbean, that are not iron limited and where algal growth is controlled by other nutrients.

Microbial ratchet. One enigmatic observation is the large distances that the black reefs extend from the shipwrecks. Since iron transport via the water would lead to mixing and dilution, we hypothesize that a remineralizing microbial-algal mat uses a ratchet-like mechanism to crawl down the reef. In this model, the microbial-algal mats on the point source scavenge iron, overwhelming the corals and CCA in the immediate area. As they expand outward, they carry iron within them that, upon their death, becomes locally available. This would enable the mat to extend farther still from the original shipwreck site and retain the higher concentration of iron. In any case, there is a fascinating sediment-microbe-algae-iron remineralization story on the black reefs awaiting future studies.

Conservation implications. Ecological disturbances that support algal growth on reefs can reach critical thresholds resulting in a shift to an alternative stable state [7, 32]. This new state is then maintained by self-reinforcing feedback mechanisms [33]. On coral reefs, it has been posited that the shift to an algal-dominated state could be irreversible even after the disturbance is resolved because a *feedback* may exist if the algae are detrimental to coral recovery [32, 34]. Possible mechanisms for this include algal influence on water clarity, nutrient availability, microbial activity, and allelopathy [12, 35, 36].

We have shown here that some of the most pristine coral reefs in the world are particularly sensitive to shipwrecks and the associated iron. These findings also have significant implications for the unpredictable problems that may result from iron fertilization projects in the ocean. Coral recovery requires substantial reproductive

success followed by prolific settlement and growth [6]. For the LI black reefs, recovery is promising after remediation of shipwreck sites because these remote reefs are spared many anthropogenic impacts, such as overfishing and pollution, and because high densities of coral cover nearby increase the likelihood of repopulation by scleractinian corals [4]. In summary, we would like to emphasize the importance of removing shipwrecks from coral reefs. These near-pristine reefs in the central Pacific should be preserved at all costs as they represent our last glimpse of one of the planet's most remarkable environments.

Materials and Methods

Benthic surveys and sample collection. The benthic community was characterized at 10 m depth on the forereef of each island using photoquadrats [3]. Two 25 m transect lines were quantified per site and ten 0.72 m^2 quadrats were assessed along each transect line using digital underwater photographs. Benthic organisms were classified using Photogrid 1.0; 100 points were randomly chosen and the organism at each point was identified to the lowest taxonomic level possible (genus level for corals, functional group for turfs and crustose coralline algae, and species level for macroalgae).

Algae and rubble samples (mixed algal communities living on carbonate skeletons) were collected at 10 m depth from the ship wreck site on Millennium (April 2009) and from control sites on Millennium and other LI (April 2009 and August – September 2005) and stored in sealed plastic bags at -20 ^oC on shipboard and at -80 ^oC in the lab. Voucher specimens of *Derbesia tenuissima* (BISH 743221) from Kingman Reef and *Gelidium isabelae* (BISH 743222) from Millennium Atoll have been archived at the Bishop Museum Herbarium Pacificum, Honolulu, HI, USA.

GIS map of oceanic iron concentrations. The data for oceanic iron concentrations were taken from Behrenfeld et al. [37], wherein concentrations were estimated based on deposition of soluble iron. Data were imported into the SeaWiFS Data Analysis System (SeaDAS) for image analysis.

Measurement of algal iron concentrations. Algae and rubble samples were decalcified using 3.7 N HCl until carbonate structures were no longer visible and

further acid addition produced no bubbling. Remaining tissues were rinsed 3X with 5 ml DI water, dried at 60 ^{o}C, and weighed on a microbalance (Mettler-Toledo Inc., Columbus, OH, USA). Tissues were then ashed for > 12 hours at 550 ^{o}C and dissolved into nitric acid (HNO_3, 0.5 N f/v). Iron concentrations were measured using inductively coupled plasma (Optima 4300 DV ICP-OES 4300, Perkin Elmer, Waltham, MA, USA). Ionized iron atoms were detected by ICP-OES at 259 nm.

Carbon and nitrogen content of algae and rubble tissue were measured using an elemental analyzer (2400 CHN/O EA, Perkin Elmer, Waltham, MA).

Iron enrichment experiments. *Pocillopora meandrina* coral samples were collected from a reference site on Tabuaeran Atoll. Individual 3 cm fragments (N = 80) were placed in 1 liter of reef water in acid-washed Nalgene polycarbonate bottles. Rubble samples were collected from the black reef site on Tabuaeran. Rubble pieces (~2 cm in diameter) were added to 40 of the aquarium bottles. Eight different treatments, ten replicates each, were carried out: coral; coral + ampicillin; coral + iron; coral + iron + ampicillin; coral + rubble; coral + rubble + ampicillin; coral + rubble + iron; coral + rubble + iron + ampicillin. For treatments, iron chloride ($FeCl_3$, Sigma, St. Louis, MO, USA) was added (1 μM final concentration); ampicillin (Sigma, St. Louis, MO, USA) was added (50 μg/ml final concentration) at time zero and refreshed after 48 hours. All treatments were incubated at ambient temperature with continuous aeration for five days. Coral fragments were photo-documented and the concentration of dissolved oxygen was measured in each aquarium.

Dissolved oxygen measurements. The concentration of dissolved oxygen (DO) was measured in each aquarium using a Hach LDO101 Standard dissolved oxygen probe (Hach, Loveland, CO, USA). The aeration tubing was removed from the aquariums ~five minutes prior to DO measurement. Measurements were made at the end of the five day treatments and approximately two hours later.

Metagenomic library construction and analysis. At each site, approximately 100 l of seawater was collected from below the boundary layer (in crevices and against the benthos) above ~20 m^2 of reef using a modified bilge pump connected to low density polyethylene (LDPE) collapsible bags (19 l; Cole-Parmer, Vernon Hills, IL, USA) and transported to the research vessel within two hours. Prior to sampling, containers, bilge pumps, and tubing were washed once with 1% bleach and 0.1 N NaOH, three times with freshwater, and once with 100 kDa filtered seawater. On shipboard the large eukaryotes were removed by filtration through 100 μm Nitex. The filtrate was then concentrated to <500 ml using a 100 kDa tangential flow filter (TFF), which captured the unicellular eukaryotes, microbes, and virus-like particles. Microbial cells were collected by passing the concentrated sample through 0.45 μm Sterivex filters (Millipore, Inc) and the filters were stored at -80 ^{o}C.

After removing the Sterivex filters, DNA was extracted by standard phenol:chloroform DNA protocols. The DNA was amplified with φ29 polymerase (Monserate biotech, La Jolla, CA, USA), four to six replicates per site. The replicates were pooled and purified using silica columns (Qiagen Inc, Valencia, CA, USA). The DNA was precipitated with ethanol, re-suspended in water (~300 ng $ml^{-1)}$, and then

pyrosequenced at the University of Carolina (Engencore) using the 454 GS-FLX platform.

Metagenomic sequences were uploaded to the MG-RAST server (http://metagenomics.nmpdr.org/metagenomics.cgi) for functional and taxonomic annotation. Sequences were compared to the SEED protein database using BLASTx [38]. Sequences with significant similarities (E-value <0.00001) were assigned functions based on their closest similarity [39] and were then grouped into metabolic pathways (subsystems). Also included in the subsequent analyses were metagenomic libraries from four atolls in the Northern Line Islands [24].

Sequence comparison to the virulence factor database (VFDB). The six metagenomic libraries were compared to the virulence factor database [29] (http://www.mgc.ac.cn/VFs/) using BLASTX (E-value <0.0001).

Microbial abundances. Direct counts of microbes (Bacteria and Archaea) were made by epi-fluorescent microscopy. Seawater samples were obtained at each site at the same time of day from 25 cm above the benthos (~10 m depth), then fixed in paraformaldehyde (2% final concentration) within one hour of collection, filtered onto 0.02 μm anodisc membrane filters (Whatman, Inc., Florham Park, NJ, USA), and stained with SYBR Gold (5x final concentration, Invitrogen, Carlsbad, CA, USA). Microbes were counted in 10 fields selected at random (>200 per sample).

Statistics. Statistical analyses were made using R [40]. The nonparametric Mann-Whitney U-test, performed using the Wilcox test procedure, was used to assess significant differences between reef sites. Non-metric multidimensional scaling

(nMDS) used the isoMDS function with a Euclidean distance matrix. A similarity profile test (SIMPROF) [41] was used to determine whether similarities observed between data sets (metabolic profiles in this study) are significant or likely arose by chance. The test was based on a Bray-Curtis similarity matrix and groupings examined at the 5% significance level (<0.05) using a maximum of 1000 random permutations of the raw data.

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Author Contributions

The manuscript was written by LK and FLR. The iron measurements and metagenomic analyses were completed by LK. The sample collection and field experiments were completed by KLB, EAD, AMF, MJAV, ES and FLR. The benthic characterizations were completed by JES and GJW. SAS and DO characterized the black reefs on Millennium, Starbuck, Tabuaeran, and the Phoenix Islands. BN constructed the iron map using GIS and helped with the bioinformatics. DW completed the statistical calculations and provided valuable computational support to LK. The SIMPROF analysis of the metabolic sequence data was completed by GJW. All of the authors offered helpful comments to the manuscript.

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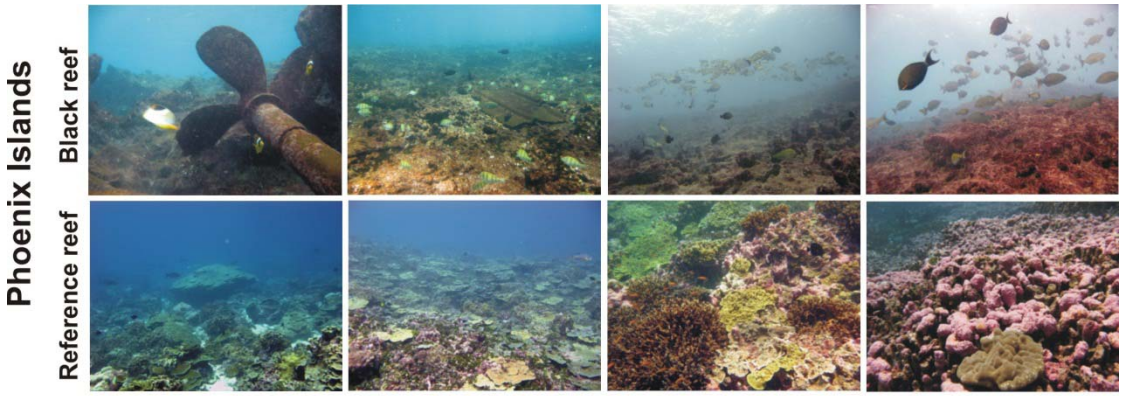
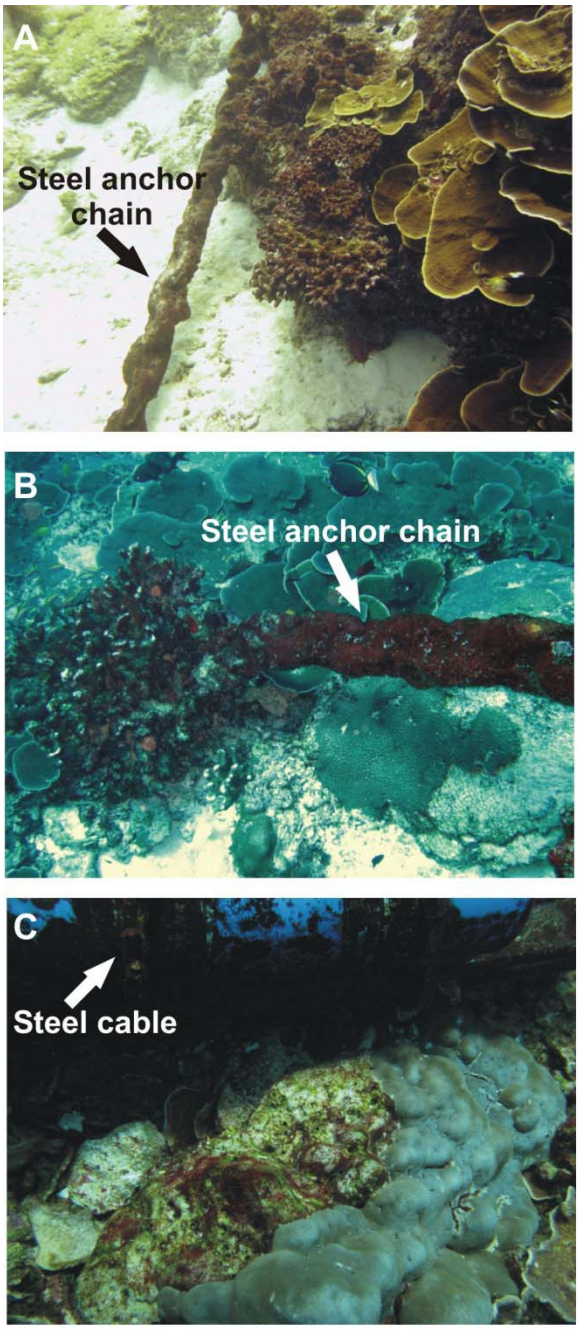
Appendix

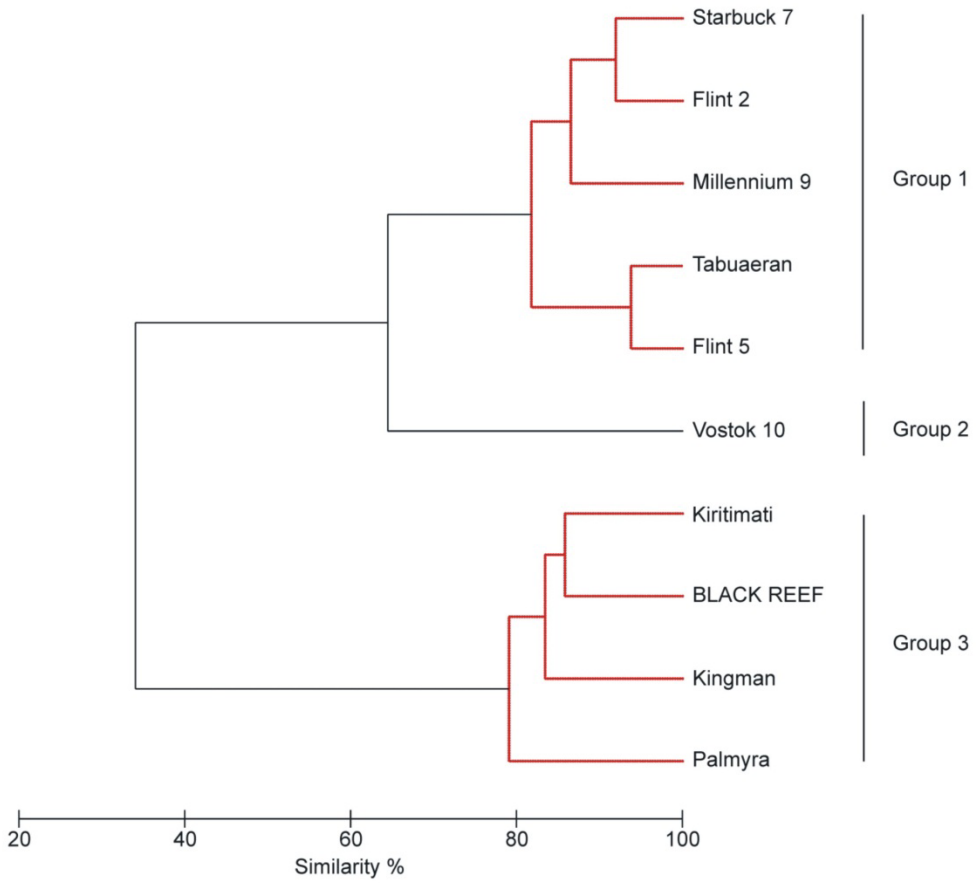
Supplementary Figures



Supplementary Figure 4.1. Representative photographs of benthic habitats from black reefs and reference reefs from the Phoenix Islands.



Supplementary Figure 4.2. Representative photographs of *mini-black-reefs*. Smaller black reefs surrounding steel anchor lines and steel cable ties located on Enderbury, Phoenix Islands (A) and Jarvis, Line Islands (B & C).

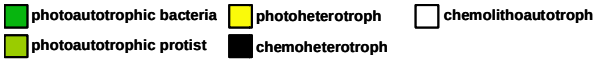


Supplementary Figure 4.3. Similarity profile test based on the metabolic profile of the microbial communities from the Black Reef and nine other coral reefs throughout the Northern and Southern Line Islands. Three significant groupings (P = <0.05) are seen.

Top ten similarities to microbial organisms

Species	No. sequence similarities	% of total SEED similarities	Trophic lifestyle	Trophic lifestyle (total bacteria + protists)
	Millennium 19 (Black reef)			
Candidatus Pelagibacter ubique	2950	19.4	chemoheterotrophic bacteria	
Prochlorococcus marinus subsp.	1092	7.2	photoautotrophic bacteria	
Prochlorococcus marinus	1011	6.7	photoautotrophic bacteria	
Flavobacteria sp.	550	3.6	chemoheterotrophic bacteria	
Flavobacteriales bacterium	366	2.4	chemoheterotrophic bacteria	
Croceibacter atlanticus	364	2.4	chemoheterotrophic bacteria	
Gramella forsetii	358	2.4	chemoheterotrophic bacteria	
Flavobacterium sp.	307	2.0	chemoheterotrophic bacteria	
Cellulophaga sp.	287	1.9	chemoheterotrophic bacteria	
Acinetobacter baumannii	277	1.8	chemoheterotrophic bacteria	
	Millennium 9			
Pseudomonas putida	207	14.3	chemoheterotrophic bacteria	
Odontella sinensis	129	8.9	photoautotrophic protist	
Methylophaga thalassica	93	6.4	chemoheterotrophic bacteria	
Nitrosomonas sp.	91	6.3	chemolithoautotrophic bacteria	
Candidatus Pelagibacter ubique	71	4.9	chemoheterotrophic bacteria	
Acinetobacter baumannii	56	3.9	chemoheterotrophic bacteria	
Nostoc punctiforme	36	2.5	photoautotrophic bacteria	
Euglena gracilis	27	1.9	photoautotrophic protist	
Trichodesmium erythraeum	24	1.7	photoautotrophic bacteria	
Thermosynechococcus elongatus	20	1.4	photoautotrophic bacteria	
	Flint 2			
Odontella sinensis	411	10.7	photoautotrophic protist	
Guillardia theta	185	4.8	photoautotrophic protist	
Euglena gracilis	129	3.4	photoautotrophic protist	
Thermosynechococcus elongatus	118	3.1	photoautotrophic bacteria	
Trichodesmium erythraeum	101	2.6	photoautotrophic bacteria	
Gloeobacter violaceus	88	2.3	photoautotrophic bacteria	
Candidatus Pelagibacter ubique	85	2.2	chemoheterotrophic bacteria	
Nostoc punctiforme	80	2.1	photoautotrophic bacteria	
Pseudomonas putida	79	2.1	chemoheterotrophic bacteria	
Cyanophora paradoxa	66	1.7	photoautotrophic protist	
	Flint 5			
Micrococcus luteus	477	21.5	chemoheterotrophic bacteria	
Zymomonas mobilis	193	8.7	chemoheterotrophic bacteria	
Acidiphilium cryptum	191	8.6	chemoheterotrophic bacteria	
Odontella sinensis	162	7.3	photoautotrophic protist	
Bacillus subtilis	146	6.6	chemoheterotrophic bacteria	
Shigella sonnei	83	3.7	chemoheterotrophic bacteria	
Microcystis aeruginosa	63	2.8	photoautotrophic bacteria	
Nostoc punctiforme	59	2.7	photoautotrophic bacteria	
Acinetobacter baumannii	53	2.4	chemoheterotrophic bacteria	
Guillardia theta	35	1.6	photoautotrophic protist	
	Starbuck 7			
Odontella sinensis	803	9.4	photoautotrophic protist	
Acinetobacter baumannii	506	5.9	chemoheterotrophic bacteria	
Candidatus Pelagibacter ubique	366	4.3	chemoheterotrophic bacteria	
Guillardia theta	296	3.5	photoautotrophic protist	
Thermosynechococcus elongatus	244	2.8	photoautotrophic bacteria	
Xanthomonas campestris	224	2.6	chemoheterotrophic bacteria	
Nostoc punctiforme	221	2.6	photoautotrophic bacteria	
Pseudomonas putida	207	2.4	chemoheterotrophic bacteria	
Euglena gracilis	153	1.8	photoautotrophic protist	
Cyanophora paradoxa	150	1.7	photoautotrophic protist	
	Vostok 10			
Acinetobacter baumannii	2599	15.7	chemoheterotrophic bacteria	
Pseudomonas putida	2155	13.0	chemoheterotrophic bacteria	
Methylophaga thalassica	1114	6.7	chemoheterotrophic bacteria	
Acinetobacter sp.	1095	6.6	chemoheterotrophic bacteria	
Nitrosomonas sp.	799	4.8	chemolithoautotrophic bacteria	
Idiomarina loihiensis	674	4.1	chemoheterotrophic bacteria	
Citrobacter freundii	634	3.8	chemoheterotrophic bacteria	
Odontella sinensis	435	2.6	photoautotrophic protist	
Vibrio splendidus	387	2.3	chemoheterotrophic bacteria	
Campylobacter hominis	358	2.2	chemoheterotrophic bacteria	

No., number. The pie charts include all sequence similarities designated by the SEED database, E-value < 0.00001.



Supplementary Figure 4.4. A list of the top ten taxonomic similarities to microbial organisms (bacteria and protists) for six Line Island reef communities. Sequences were compared to the SEED database, E-value <0.00001. The pie charts represent the trophic lifestyle for the total number of microorganisms with significant sequence similarities. The total number of organisms used for these charts are as follows; Black reef = 14096, Millennium 9 = 1176, Flint 2 = 2410, Flint 5 = 1949, Starbuck 7 = 6363, Vostok 10 = 15327.

Supplementary Tables

Supplementary Table 4.1. Elemental iron concentrations in mixed algae determined using ICP.

Sample Name	Location	Type of algae	µg Fe / g dry algae (ppm)	Fe µmols ($\times 10^{-1}$)	C mols ($\times 10^{-3}$)	µmol Fe : mol C
CARSW0422A9	Black reef	Turf / Cyano	**1304.09**	2.8	0.43	**647.63**
CARSW0422R1	Black reef	Turf / Cyano	**1054.82**	7.18	1.13	**633.07**
CARSW0422R8	Black reef	Turf / Cyano	**474.27**	4.08	1.71	**238.34**
CARSW0422R7	Black reef	Turf / Cyano	**695.51**	7.22	1.89	**382.32**
CARSW0422R10	Black reef	Turf / Cyano	**2758.22**	8.89	0.57	**1565.4**
CAR0419R5	Millennium	Mixed red	**387.97**	1.11	0.28	**393.35**
CAR0419R7	Millennium	Mixed red	**132.41**	0.68	0.69	**98.63**
CAR0417R2	Millennium	Mixed red	**148.87**	0.32	0.25	**128.2**
CAR0417R4	Millennium	Mixed red	**99.08**	1.33	1.84	**72.32**
CAR0417A1	Millennium	*Halimeda opuntia*	**15.97**	0.15	2.09	**7.01**
CAR0417A5	Millennium	*Chlorodesmis* sp.	**231.62**	1.04	0.94	**110.83**
MAL0411R2	Malden	Mixed red	**140.56**	1.01	ND	**ND**
VOS0401R2	Vostok	Mixed red	**158.27**	0.45	0.54	**82.93**
VOS0401R3	Vostok	Mixed red	**103.55**	0.63	1.07	**58.86**
VOS0401R5	Vostok	Mixed red	**113.49**	1.08	1.51	**71.28**
KIRB11A1	Kiritimati	*Halimeda opuntia*	**192.9**	0.38	0.35	**108.42**
KINGB6A4	Kingman	*Halimeda opuntia*	**103.51**	0.46	0.49	**93.25**
NA	Davies Reef	*Boodlea composita*	**85**			Entsch et al. 1983
NA	Davies Reef	*Ceramium gracillimum*	**17**			Entsch et al. 1983
NA	Davies Reef	*Chlorodesmis comosa*	**105**			Entsch et al. 1983
NA	Davies Reef	filamentous turf	**160**			Entsch et al. 1983
NA	Davies Reef	cyanobacteria	**300-400**			Entsch et al. 1983

ppm, parts per million. ND, not determined, GBR, Great Barrier Reef.

CHAPTER 5: Conclusions

Studies that incorporate the influence of microbial dynamics on reef ecosystems are essential to better understand the mechanisms by which algae are able to overgrow or kill reef-building corals. This dissertation has provided a brief overview of what is known about coral-associated microbes, as well as three metagenomic studies characterizing the microbes inhabiting coral reefs. Chapter 5 summarizes the results presented in this dissertation and offers suggestions for future work to better understand the influences of microbes in coral reef ecosystems.

Summary of results from this dissertation

The coral holobiont in health and disease. Corals benefit from associations with their microbial symbionts. In chapter 2, the significance of the microbial symbionts within the coral holobiont involved in recycling nitrogen was highlighted. Here it was demonstrated that in addition to bacteria and archaea, the endolythic fungi possess the genetic potential for the nitrogen remineralization via dissimilatory reduction of nitrate to ammonia (DRNA) and assimilation of ammonia for biosynthesis. Therefore the fungal symbionts associated with corals represent a previously undescribed microbial contributor to the holobiont nitrogen budget.

The community metabolism of the microbes associated with a healthy coral demonstrated the importance of nutrient recycling and reflected the capability to degrade the complex carbon sources of which coral mucus is composed (Chapter 2). One implication of altering the microbes associated with the coral host is selecting against beneficial microbial symbionts. Coral subjected to environmental stressors

underwent shifts in microbial community composition where microbes associated with healthy corals were replaced by microbes associated with both bacterial and fungal diseases [1]. When a coral is afflicted with disease, healthy symbionts that increase the overall fitness of the collective holobiont (e.g., providing nutrients) may be outcompeted by pathogenic microbes [2]. Kimes and colleagues [3] showed significant changes in microbial community metabolism between healthy and Yellow Band Disease states, though counter to the above argument, the abundance of genes involved in nitrification increased in diseased tissues. Nevertheless, there is still much to understand regarding the roles of the microbial counterparts in a healthy coral holobiont as well as the changes that occur during disease [4].

Adaptations to the microbial community reflect local nutrient concentrations. The community metabolism of coral-associated microbes inhabiting coral surfaces and within the boundary layer was shown to reflect the local availability of nutrients (Chapter 3). Greater nutrient availability was associated with more metabolic versatility whereby these microbes had an increased capacity for nutrient transport, motility and chemotaxis, as well as transfer of genetic material via conjugation (Summarized in Figure 5.1). Adaptation to increases in local nutrient concentrations was also observed in the microbial metagenomes from the black reef (Chapter 4). Here, virulence genes related to iron were an order of magnitude higher in the black reef metagenome compared to reference reefs. I hypothesized that these microbes were adapting to local conditions via horizontal gene transfer of advantageous metabolic genes. However, this question remains to be investigated in future studies.

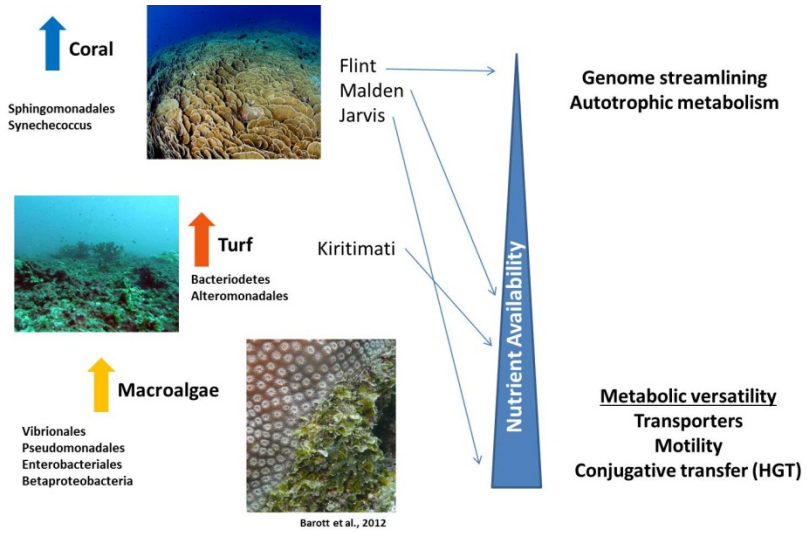


Figure 5.1. Summary of the predictor variables influencing microbial community structure and metabolism.

Primary carbon sources influence microbial community structure. The microbial growth dynamics and community composition on coral reefs are influenced by the percent cover of benthic primary producers. A series of studies was conducted on the lagoonal reefs of Mo'orea, French Polynesia determined the influence of algal-derived organic exudates on reef associated microbial communities. Rates of photosynthesis, respiration, and dissolved organic carbon (DOC) release were assessed for six of the most common benthic reef organisms from the backreef habitat. The microbial community response to dissolved exudates of each benthic producer was subsequently examined in two-day dark remineralization incubations by measuring bacterioplankton growth, respiration, and DOC drawdown. The results showed that all five types of algae measured, but not the coral, exuded significant amounts of labile DOC into their surrounding environment. For example, turf algae produced nearly twice as much DOC per unit surface area as the other benthic producers (14.0 ± 2.8

μmol h^{-1} dm^{-2}), stimulating rapid bacterioplankton growth (0.044 ± 0.002 log10 cells h^{-1}) and concomitant oxygen drawdown (0.16 ± 0.05 μmol L^{-1} h^{-1} dm^{-2}, Figure 5.2). Therefore, different benthic primary producers contribute significantly to microbial ecology by manipulating key environmental parameters (i.e., DOC and oxygen O_2 availability), which influences bacterial abundance and metabolism on coral reefs.

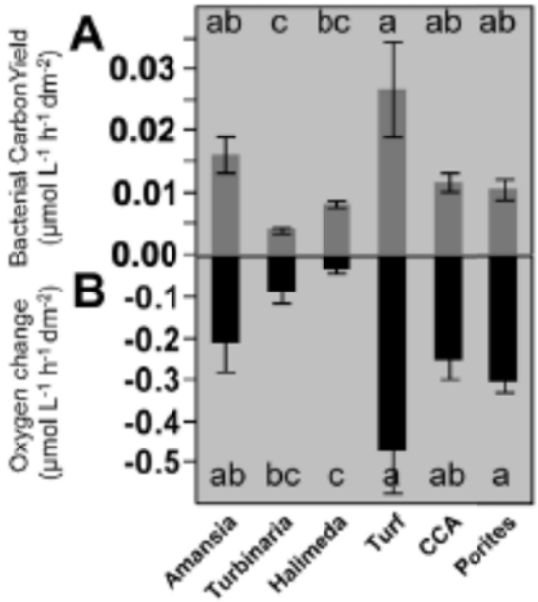


Figure 5.2. Rates of bacterial carbon yield (A) and DO consumption on exudates over 48 hour dilution culture incubations of previously incubated primary producers (B). Bars are means with standard error whiskers; treatments with the same letter are not significantly different at a = 0.05. (Published in Haas et al., [5])

Chapter 3 demonstrated that the percent cover of benthic primary producing macrobes correlated with specific bacterial groups. The distribution of microbial taxa was most strongly correlated with the prevalence of certain benthic functional groups. For example, reefs with higher coral cover observed on Islands Malden, Flint, and Vostok were associated with higher abundances of *Sphingomonadales* (Figure 5.1). In contrast, Kiritimati reefs which were dominated by turf algae (58.9-82.4%) and had

very little coral cover, were associated with higher abundances of *Bacteriodetes*. The reefs on Tabuaeran and Teraina had higher presence of macroalgae and were associated with greater abundances of *Gammaproteobacteria* (specifically, Orders *Pseudomonadales* and *Enterobacteriales*) and *Betaproteobacteria*. The results of this study suggest that coral reef associated microbes are selected for by the carbon sources exuded by the benthic primary producers.

This observation is supported by an empirical investigation of the influence of the composition of benthic reef exudates on reef associated microbes. Here, coral and algal exudates were further characterized to elucidate DOC composition based on fractional dissolved combined neutral sugars (DCNS). Microbial community responses to each exudate were subsequently tracked over 48 hours to assess cellular growth, DOC/DCNS utilization, and changes in taxonomic composition (via 16S rRNA amplicon pyrosequencing). Fleshy macroalgal exudates were enriched in the DCNS components fucose and galactose whereas coral and coralline algal exudates were enriched in total DCNS but in the same component proportions as ambient seawater (Figure 5.3). Rates of microbial growth and DOC utilization were significantly higher in algal exudate treatments than in coral exudate and control incubations with each community selectively removing different DCNS components. Coral exudates engendered the smallest shift in overall bacterioplankton community structure, maintained high diversity, and enriched taxa from *Alphaproteobacteria* lineages containing cultured representatives with relatively few virulence factors (*Hyphomonadaceae*, *Erythrobacteraceae*). In contrast, macroalgal exudates selected

for less diverse communities heavily enriched in copiotrophic *Gammaproteobacteria* lineages containing cultured pathogens with increased virulence factors (*Vibrionaceae*, *Pseudoalteromonadaceae*; Figure 5.3). In a similar mesocosm experiment, turf algal exudates also enriched for *Bacteriodetes* bacteria from the Order *Flavobacteria* [6].

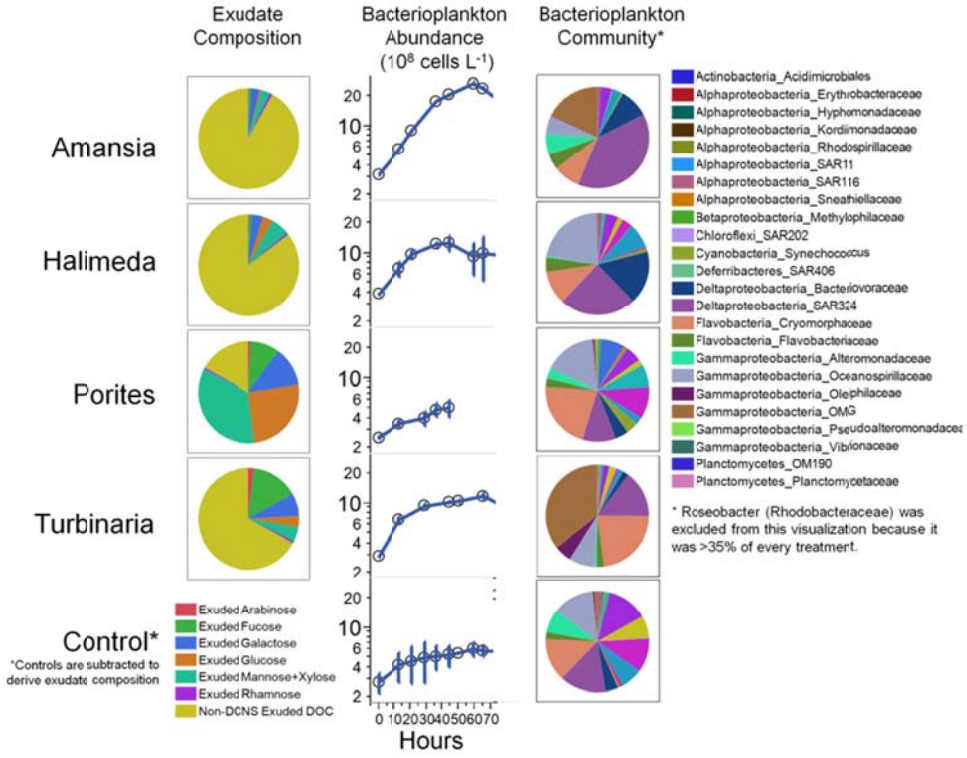


Figure 5.3. Summary of differences in exudate composition, subsequent bacterioplankton growth, and resulting bacterial community structure among the experimental treatments (Published in Nelson et al. [7]).

Collectively, these three studies provide compelling evidence that algal-derived DOM promotes increased activity and alters community composition of coral-associated microbes. Furthermore, the Haas et al. [5] and Nelson et al. [7] studies provide a mechanism for why different species of algae are more detrimental to corals than others; that is 1) differences in release rates and 2) distinct composition of DOM.

Microbial mediated feedbacks on coral reefs. These studies support a model that predicts that coral-algal phase shifts result in more algal-released DOM and that these increased energy inputs stimulate the growth and development of 1) microbes that cause both non-specific diseases (hypoxia) and 2) opportunistic pathogens that promote mortality and disease on coral reefs.

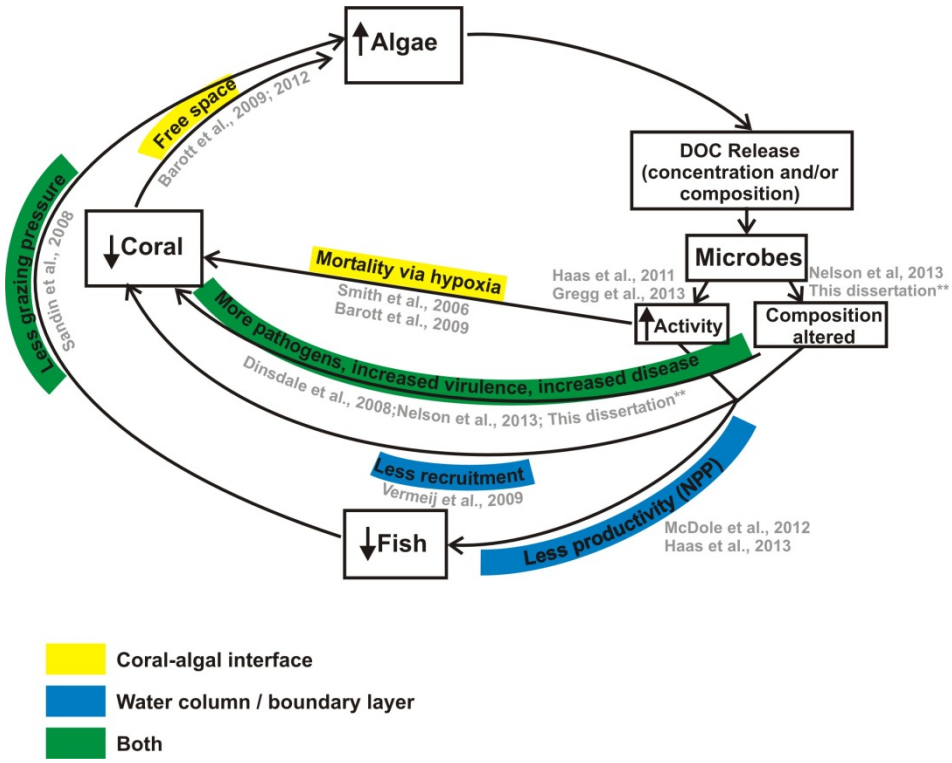


Figure 5.4. Summary of biological feedbacks on coral reefs.

Figure 5.4 summarizes the positive feedback loop which results in the loss of coral cover on reefs. Several studies have demonstrated that eutrophication and overfishing support the overgrowth of algae over corals. For example, in the Line Islands, Sandin et al., [8] showed that coral reef degradation was largely influenced by fishing pressure. These conditions that shift the competitive advantage towards algae

result in a positive feed-back loop which involves the microbial community. Here, increased abundances of algae exudes DOC onto the reef which stimulates microbial activity of coral associated microbes, particularly at coral-algal interfaces [9] and in the water column [5]. This increased activity of coral-associated microbes results in concomitant oxygen drawdown which causes hypoxia in the coral tissues and leads to mortality [10, 11]. Damage to coral colonies and coral mortality at coral-algal borders results in the availability of free space on the reef, which quickly becomes colonized by new algae [11, 12]. Algal derived DOC also alters the composition of the microbial community on reefs [7, 13]. Higher coverage of benthic algae and subsequent increased availability of algae derived DOC elicits a community shift in coral reef microbes response towards a community dominated by "super" heterotrophs containing more potential pathogens [14] with higher abundances of virulence factors [7, 15]. This shift in microbial community composition is associated with increased prevalence of coral disease [8]. These microbial dynamics on coral reefs have far reaching repercussions having been shown to negatively influence coral recruitment [16] as well as decrease the primary productivity of the reef ecosystem as a whole [17], thereby providing less energy which influences the biomass of organisms at higher trophic levels of the food web such as fish [18]. Less fish results in higher abundances of benthic algae, thus the cycle continues. This doctoral research has contributed to the understanding of some aspects of this biological positive feedback model including the influences of DOC concentration and composition on microbial activity as well as microbial community composition both *in situ* and in microcosms,

and the increased presence of virulence genes and potential pathogens on algae dominated reefs.

Future studies

What is the mechanism for the observed trend in community metabolism? One aspect of this research that is unclear is whether the metabolic genes observed to correlate with nutrient availability are moving horizontally through the community or whether strains of microbes with the advantageous genes are becoming more abundant (beta diversity).

Predicting reef microbes. In order to devise effective coral reef management strategies, we need to continue to elucidate the mechanisms by which algae are able to overgrow or kill reef-building corals. Given the growing body of evidence linking coral mortality with microbes and algal-derived DOC, it is essential to identify the key microbial players driving this process. My research has demonstrated that taxonomic composition was most strongly correlated with the composition of benthic primary producers on the reef (Chapter 3). Furthermore, the Haas et al. [5] and Nelson et al. [7] studies collectively demonstrate that benthic primary producers differentially influence microbial activity and community structure on coral reefs. These patterns are consistent both experimentally in microcosms [7] and *in situ* (Chapter 3). Therefore it may be possible to use photographs of benthic reef communities in order to predict the microbial community dynamics on the reef. Benthic composition surveys are a standard protocol for reef monitoring programs whereas the procedures for microbial sampling require specialized equipment and sample processing that is both time

consuming and costly. The ability to make predictions about the microbial communities is essential for understanding long term trajectories of coral reefs.

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