UNIVERSITY OF CALIFORNIA, SAN DIEGO

Ecological significance of bacteria associated with coral reef fish feces

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Marine Biology

by

Steven Paul Smriga

Committee in charge:

Professor Farooq Azam, Chair
Professor Douglas Bartlett
Professor Partho Ghosh
Professor Phil Hastings
Professor Jennifer Smith

2010

The dissertation of Steven Paul Smriga is approved, and it is acceptable in the quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____

Chair

University of California, San Diego

2010

TABLE OF CONTENTS

SIGNATURE PAGE ..... **iii**

TABLE OF CONTENTS ..... **iv**

LIST OF FIGURES ..... **vi**

LIST OF TABLES ..... **viii**

ACKNOWLEDGEMENTS ..... **ix**

VITA ..... **xii**

ABSTRACT OF THE DISSERTATION ..... **xiii**

CHAPTER 1: Introduction **1**

REFERENCES 8

CHAPTER 2: Gradients of coastal fish farm effluents and their effect on coral reef microbes **10**

SUMMARY 11

INTRODUCTION 11

RESULTS 12

DISCUSSION 16

EXPERIMENTAL PROCEDURES 20

REFERENCES 23

CHAPTER 3: Abundance, diversity, and activity of microbial assemblages associated with coral reef fish guts and feces **26**

ABSTRACT 27

INTRODUCTION 27

MATERIALS AND METHODS 28

RESULTS 30

DISCUSSION 32

REFERENCES 37

CHAPTER 4: Persistence of parrotfish feces derived bacteria in coral reefs **40**

ABSTRACT 41

INTRODUCTION 42

MATERIALS AND METHODS 44

RESULTS 48

DISCUSSION....51
REFERENCES....61

CHAPTER 5: Effects of parrotfish feces deposition on corals and coral-associated microbial assemblages....63

ABSTRACT....64
INTRODUCTION....65
MATERIALS AND METHODS....67
RESULTS....74
DISCUSSION....80
REFERENCES....95

CHAPTER 6: Use of ethynyldeoxyuridine incorporation and click chemistry to detect DNA synthesis in marine bacteria....97

ABSTRACT....98
INTRODUCTION....99
MATERIALS AND METHODS....101
RESULTS....109
DISCUSSION....113
REFERENCES....127

CHAPTER 7: Conclusions....130

REFERENCES....133

LIST OF FIGURES

Figure 2-1: Bacteria abundances and particle abundances in water collected from sites along a transect near fish farms 13

Figure 2-2: DGGE profiles for 16S rRNA genes of free-living and particle-attached bacteria in water near fish farms 14

Figure 2-3: Representative microscopy images of particle-attached and free-living bacteria in water near fish farms 14

Figure 2-4: Distribution of phyla in 16S rRNA gene clone libraries from water, coral, and fish feces 15

Figure 2-5: Percent of novel sequences in 16S rRNA gene clone libraries from water, coral, and fish feces 16

Figure 2-6: Location of study sites near Bolinao, Pangasinan, Philippines 20

Figure 3-1: Clone library and DGGE results for 16S rRNA genes from feces of three fishes 32

Figure 3-2: Phylogenetic trees of 16S rRNA genes for *Vibrio* spp. and *Photobacterium* spp. from fish feces 33

Figure 3-3: Phylogenetic tree of 16S rRNA genes from fish feces highlighting unique fish feces/fish gut taxonomic group 35

Figure 4-1: Representative DGGE profiles for multiple parrotfish feces highlighting two common band. 59

Figure 5-1: Dry weights of individual feces from *Chlorurus sordidus* by fish length and mean size-class-specific feces mass and flux 87

Figure 5-2: 16S rRNA gene DGGE profiles for coral mucus, aged parrotfish feces, and feces-mucus-bundles from *in situ* incubations of *Montipora aquituberculata* coral. 88

Figure 5-3: Design for aquaria experiments where coral fragments were challenged with parrotfish feces. 89

Figure 5-4: 16S rRNA gene DGGE profiles for blastates of *Montipora aquituberculata* coral fragments held in aquaria experiments for 92 h. 91

Figure 5-5: 16S rRNA gene DGGE profiles for blastates of *Acropora hyacinthus* coral fragments held in aquaria experiments for 96 h. 92

Figure 5-6: 16S rRNA gene DGGE profiles for bacterial assemblages associated with settled particles accumulated in the bottom of aquaria over 96 h. 93

Figure 5-7: Seawater bacteria abundances in experimental aquaria.. 94

Figure 6-1: Overview of protocol for ethynyldeoxyuridine (EdU) incorporation into marine bacteria and detection using click chemistry. 121

Figure 6-2: Epifluorescence microscopy images of EdU-labeled bacteria from coastal seawater and stonefish feces. 122

Figure 6-3: Time course showing bacterial cell abundance and protein production in coastal seawater at four concentrations of EdU. 123

Figure 6-4: Time course showing total cell abundance, EdU-labeled cell abundance, and percentage EdU-labeling in seawater 124

Figure 6-5: Localization of signal in EdU-labeled bacteria. 126

LIST OF TABLES

Table 2-1: Chemical characteristics of water collected from sites along a transect near fish farms 12

Table 2-2: Microbial abundances and particle abundances in water collected from sites along a transect near fish farms 13

Table 2-3: List of 16S rRNA gene sequences with high similarity to genes previously reported from shallow-water coral samples 17

Table 2-4: Highly similar 16S rRNA genes from different sample sites along a water transect 18

Table 3-1: Bacteria abundances and hydrolytic enzyme rates associated with *Chlorurus sordidus* 31

Table 4-1: Description of samples collected from Palmyra Atoll.....56

Table 4-2: Results for BLAST comparison of fish feces vibrio-like (FFV-L) 16S rRNA gene to metagenomic databases.....57

Table 4-3: PCR amplification from several feces using FFV-L specific primer 57

Table 4-4: Identities of individual DGGE bands from parrotfish feces Presence or absence of FFV-L in coral mucus after transplanting parrotfish feces onto coral surfaces 58

Table 4-5: Identities of individual DGGE bands from parrotfish feces 60

Table 6-1: Specific growth rates and individual cell EdU signal intensity for four bacterial isolates ...125

ACKNOWLEDGEMENTS

There are many people who supported me during work on this dissertation. I start by thanking my advisor Farooq Azam and dissertation committee members Doug Bartlett, Partho Ghosh, Phil Hastings, and Jen Smith for helpful advice and critical reading of the work.

I thank Stuart Sandin for the invitations to join his field trips to Palmyra Atoll and the Line Islands. Three chapters of the thesis are based on samples collected and work performed at Palmyra. His wisdom and infectious enthusiasm broadened my understanding of coral reef ecology and helped shape my studies.

I thank all of the graduate students (Melissa Garren, Francesca Malfatti, Xavier Mayali, Byron Pedler, and Ty Samo), the postdocs (Christine Beardsley, Anne-Claire Bordeaux, Krystal Rypien, Jessica Ward, and Alexandra Worden), and undergraduate assistants (Johnny Nguyen, Joseph Villareal, and Tony) who shared the collective space known as the Azam Lab over my years as a grad student. Their assistance with numerous projects and discussions that covered wide ranges of topics were a major part of my day-to-day life. Together we experienced the rewards and tribulations of science.

For helpful discussions and advice I thank and acknowledge: Eric Allen, Lihini Aluwihare, David Bourne, Greg Dick, Emiley Eloe, Jed Fuhrman, Scott Hamilton, Davey Kline, Nancy Knowlton, Mike Latz, Federico Lauro, Kristen Marhaver, Gary Moy, Ryan Mueller, Priya Naraginsao, Olga Pantos, Sheila Podell, Nicole Price, Julie Robidart, Forest Rohwer, Melissa Roth, Milton Saier, Koty Sharpe, Jennifer Smith,

and Vera Tai, among others. For their encouragement and advice before my time as a graduate student, I thank and acknowledge Drs. Philip Bond, William Stringfellow, and Thomas Duda.

For their emotional support, character building, and entertainment value I'd like to acknowledge friends that loosely group into the Supers (Kristen, Phil, Tali, Sach, Andrea, Jerry, Ty), the O'Briens-ers (Travis, Roman, Roberta, Evan, Ryan, Brian, Andy, etc.) and the Cohort (Nacho, Loren, Sheila, Jessica [x3], Jess B, Jeremy, Elisa, Dana, Melania, etc.), as well as many, others. I'd also like to thank my mother Judy, brother Scott, and father Paul for their continued support over more than a decade of higher education.

Extra special acknowledgement goes to Rachel Borgatti, who finished two master's degrees while supporting me with boundless love throughout all my time at SIO, and who shares with me in parental duties of one happy baby named Avicennia.

Finally, I'd like to acknowledge my advisor Farooq Azam. He provided: unmatched wisdom and guidance; a model for logical and critical thought; indispensable career advice; inspiration and encouragement; an always open ear and office door with thoughtful answers to questions ranging from the detailed to the absurd; a consistent sense of humor that made working long hours enjoyable; lambchops (occasionally), cake (sometimes), and coffee (often).

Chapter 1, in its entirety, is a reprint of the material as it appears in: Garren M, Smriga S, and Azam F (2008) Gradients of coastal fish farm effluents and their effect on coral reef microbes. *Environmental Microbiology* **10**: 2299-2312. The dissertation author was one of two primary investigators and a co-first author of this manuscript.

Chapter 2, in its entirety, is a reprint of the material as it appears in: Smriga S, Sandin S, and Azam F (2010) Abundance, diversity, and activity of microbial assemblages associated with coral reef fish guts and feces. *FEMS Microbiology Ecology* **73**: 31-42. The dissertation author was the primary investigator and principal author of this manuscript.

Chapter 6 is currently being prepared for submission for publication: Smriga S, Samo T, and Azam F (in prep) Use of ethynyldeoxyuridine and click chemistry to detect DNA synthesis in marine bacteria. The dissertation author was the primary investigator and principal author of the manuscript.

VITA

2000	B.S., Bacteriology, Molecular Biology University of Wisconsin, Madison
2000-2001	Research fellow, Lawrence Berkeley Laboratory
2001-2002	Research fellow, Smithsonian Tropical Research Institute
2002-2003	Research assistant, Meridian Institute, Washington DC
2010	PhD, Oceanography, Scripps Institution of Oceanography University of California, San Diego

PUBLICATIONS

Smriga, S., T. Samo, J. Villareal, and F. Azam. (*in prep*) Use of ethynyldeoxyuridine incorporation and click chemistry to detect DNA synthesis in marine bacteria.

Smriga, S., S. Sandin, and F. Azam. (2010) Abundance, diversity, and activity of microbial assemblages associated with coral reef fish guts and feces. *FEMS Microbiology Ecology* **73**: 31-42.

Garren, M.*, **S. Smriga***, and F. Azam. (2008) Gradients of coastal fish farm effluents and their effect on coral reef microbes. *Environmental Microbiology* **10**: 2299-2312. (*co-first authors)

Dinsdale E.A., Pantos O., **Smriga S.**, Edwards R.A., Angly F., et al. (2008) Microbial ecology of four coral atolls in the Northern Line Islands. *PLoS ONE* **3**: e1584. doi:10.1371/journal.pone.0001584

Smith, J.E., M. Shaw, R.A. Edwards, D. Obura, O. Pantos, E. Sala, S.A. Sandin, **S. Smriga**, M. Hatay, and F.L. Rohwer. (2006) Indirect effects of algae on coral: algae-mediated, microbe-induced coral mortality. *Ecology Letters* **9**: 835-845.

Worden, A.Z., M. Seidel, **S. Smriga**, A. Wick, F. Malfatti, D. Bartlett, and F. Azam. (2006) Trophic regulation of *Vibrio cholerae* in coastal marine waters. *Environmental Microbiology* **8**: 21-29.

Bond, P.L., **S.P. Smriga**, and J. Banfield. (2000) Phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. *Applied and Environmental Microbiology* **66**: 3842-3849.

ABSTRACT OF THE DISSERTATION

Ecological significance of bacteria associated with coral reef fish feces

by

Steven Paul Smriga

Doctor of Philosophy in Marine Biology

University of California, San Diego, 2010

Professor Farooq Azam, Chair

Bacteria may play important roles in the biogeochemical cycling of coral reef fish feces and in the interactions between fishes and corals. This interaction potential was observed in a study of milkfish (*Chanos chanos*) aquaculture farms in the Philippines. Effluents from suspended fish pens created steep gradients of particulate organic carbon and other water characteristics that extended into nearby coral reefs. Highly similar bacterial phylotypes co-occurred in milkfish feces and in corals indicating the potential for transport of fecal particles and interaction with corals.

In a separate study at Palmyra Atoll, bacteria abundances ranged ~10^9 to 10^{11} g^{-1} dry wt among feces of parrotfish (*C. sordidus*), snapper (*Lutjanus bohar*), and surgeonfish (*Acanthurus nigricans*). Bacteria in parrotfish feces grew at a rate of ~2 × 10^8 cells g^{-1} dry wt feces h^{-1}. To improve our ability to observe growing marine

bacteria, I tested a method for using the thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU), which becomes incorporated during DNA synthesis and can be detected using 'click' chemistry combined with epifluorescence microscopy. The percentage of EdU-labeled bacteria ranged from ~6% to 18% during a time course incubation of natural seawater assemblages. Additionally, cell specific signal intensities could be quantified, demonstrating the method's potential for determining individual cell growth rate.

Other studies addressed phylotype composition of feces-associated assemblages. Analyses of feces-derived 16S rRNA gene clones revealed that Vibrionaceae dominated parrotfish and snapper feces. Many of these genes clustered phylogenetically to cultured *Vibrio* spp. and *Photobacterium* spp. Other Vibrionaceae-like sequences comprised a distinct phylogenetic group that may represent 'feces-specific' taxa. PCR primers specific to this 'fish feces vibrio-like' group (FFV-L) were used to screen 'aged' parrotfish feces. FFV-L could be detected in feces collected over several days, indicating that feces may permit persistence of FFV-L in reefs. In addition to FFV-L, other bacteria phylotypes consistently occurred in aged feces, as determined via denaturing gradient gel electrophoresis (DGGE) analyses. To test the responses of coral-associated bacteria during short-term interactions with feces, both *in situ* and aquaria experiments were performed whereby corals were challenged with acute feces doses. The results reinforce the potential for bacteria transfer between feces and corals. Meanwhile, acute challenges with parrotfish feces did not impair the overall health of three coral species.

CHAPTER 1: Introduction

This thesis explores the response of coral-associated bacteria communities to the input of organic matter and exogenous bacteria in the form of fish feces. By using molecular techniques to describe coral- and feces-associated bacterial communities, it identifies patterns in taxa composition and physiological activity that may affect the structure of the coral holobiont as well as influence coral health and ecosystem function.

Coral reefs are threatened ecosystems and their preservation has become a societal priority. Studies continue to strengthen our knowledge of macroscale coral reef ecology, and importantly, document the effects of anthropogenic activity on coral reefs. Through these studies we are learning that fishing and other activities are causing unprecedented changes in coral and fish community composition (e.g. Sandin et al. 2008) with subsequent effects on ecological processes. Building on these macroscale studies, our knowledge of coral reef ecology, and our ability to make informed conservation and management policy decisions, will benefit from an improved understanding of the biogeochemical mechanisms that effect reef health. We know much about how microbes drive biogeochemistry in the open ocean and yet we are unable to accurately model microbially-mediated processes in order to predict how coral reef ecosystems will respond to acute or chronic human-induced stressors, especially global climate change.

My interest in studying fish feces is motivated by our relatively weak understanding of its contribution to coral reef biogeochemical processes. It is also influenced by our knowledge of marine particulate organic matter (POM) in the open

ocean. Marine POM can fall into several descriptive categories (e.g. phytodetritus, marine snow, fecal pellets) and can be derived from a wide variety of sources (e.g. phytoplankton, zooplankton exudates, abiotic physical aggregation processes, etc.)(Verdugo et al. 2004). Carbon and nutrients that comprise POM can be exported from the photic zone via sinking but most is retained in surface waters via biologically-mediated process. This process (the 'biological pump') is of long-standing interest to the field of oceanography, especially given the importance of carbon cycling to climate change. Decades of research support the hypothesis that bacteria associated with marine POM are integral to remineralization (e.g. Bidle and Azam, 2002). Furthermore, microscale interactions between bacteria and POM influence the molecular architecture of organic matter, and this contributes to large-scale biogeochemical processes (Azam and Malfatti, 2007). While our knowledge of the sources and fate of POM in the open ocean is vast, we have relatively little understanding of whether these concepts can be accurately applied to analogous processes in 'specialized' coastal ecosystems such as coral reefs.

Fish feces is a form of POM, and in coral reefs, benthic organisms may experience a persistent rain of fecal organic particles. Fish feces is mostly comprised of carbon and nitrogen, but it can also contain silicon, phosphorous, and iron as well as other nutrients that may be incorporated into detritivore biomass (Geesey et al., 1984). Fecal pellets from most herbivorous and carnivorous coral reef fishes are consumed by coprophagous fishes (Robertson 1982). Hence feces partially support marine food webs via coprophagy. However, some types of fish feces are not

consumed. They settle to the bottom, which effectively returns and redistributes POM to the benthic milieu from which it originated.

Among the various functional groups of reef fishes, parrotfish (family Scaridae) and other herbivores are especially prodigious producers of feces. For example, estimated bioerosion rates (i.e. fecal exudation rates) for at least one parrotfish species (*Chlorurus gibbus*) were 1018 kg ind^{-1} yr^{-1} (Bellwood 1995). Parrotfishes are widely distributed among global reefs, and they are keystone species whose feeding behaviors impact reef structure. Their diets include epi- and endolithic algae and their feeding behaviors are characterized by scraping of dead coral and crustose coralline algae. They ingest carbonaceous fragments and produce feces that are high in inorganic content but low in protein and lipids relative to piscivorous and zooplanktivorous fishes (Bailey and Robertson 1982). Their feces sink quickly upon egestion and are rarely consumed by other fishes (Robertson 1982). Similar to other grazing herbivores, Scarids move from one location to the next and their defecation can redistribute biomass including viable algal fragments (Smith and Smriga, in prep) and *Symbiodinium* spp. dinoflagellates (Porto et al., 2008). Importantly, parrotfish fecal pellets can settle onto corals. These traits make parrotfish feces an ecologically relevant model for studying potential interactions between fecal and coral bacteria.

Bacteria and Archaea exist in the guts and feces of marine fishes, though few studies have addressed the topic (e.g. van der Maarel et al. 1998). Because some marine bacteria are pathogenic, there is potential for fish feces to act as vectors for infectious marine diseases. For example, *Vibrio cholerae* can proliferate as free-living

cells in seawater but can also be an opportunistic human pathogen transmitted through human feces. Functional activities of feces-associated bacteria in the environment may be diverse. Jacobsen and Azam (1984) tested the hypothesis that bacteria colonize and degrade copepod fecal pellets. They found that feces were rapidly colonized and that attached bacteria contributed to pellet remineralization. A related study (Lawrence et al. 1993) found that some bacteria ingested by copepods remained viable after gut passage and that bacteria enhanced enzymatic activity in egested feces. Indeed, bacteria attached to pelagic POM express relatively high hydrolytic enzyme activities, and an ecological function of particle-associated bacteria is enzyme-mediated remineralization (Smith et al. 1992; Bidle and Azam, 1999). As we continue to explore feces-associated bacteria, we should learn the degree to which they perform parallel ecological functions as POM-associated bacteria.

The ability to estimate bacterial cell growth (Fuhrman et al. 1982) led to an expansion of our understanding for the roles of bacteria in carbon cycling in the ocean. Previously described methods to observe growing bacteria utilize radiolabeled substrates (Fuhrman et al. 1982) or require extensive processing time (Hamasaki et al., 2004). The recent emergence of the use of 'click' chemistry in the study of eukaryotic cell biology held promise of adaptation for use in microbial ecology. As described in this thesis, it enabled us to develop an alternative, rapid method that can be used to observe growth in seawater and holds great potential for determining growth in feces- and coral-associated assemblages.

The click chemistry-based technique for individual cell growth makes progress toward 'interaction biology,' i.e. adapting and developing methods to observe individual microbes interacting with each other and with their microenvironments. The ability to interrogate coral microbes at the microscale enables a framework for formulating hypotheses about the functions and processes of these communities. For example, heterogenous microenvironments in feces and corals may host actively growing cells, and these can be observed using laser confocal microscopy combined with the click chemistry-based method. The concerted exploration of community dynamics and microscale interactions is a powerful approach, and this thesis illustrates how it can lead to novel insights on how coral mucus communities respond to the inputs of exogenous organic matter and bacteria.

The conceptual context described above guided the experimental design and synthesis of results for the studies herein. The chapters of this thesis are presented as discrete studies but all follow the central theme that coral reef bacteria communities respond to the input of organic matter and exogenous bacteria in the form of fish feces.

Chapter 2 describes microbial communities in coral reefs near intensive aquaculture facilities in the Philippines. The suspended cage farms of milkfish (*Chanos chanos*) created water column gradients of chlorophyll, DOM, and POM that extended into nearby coral reefs. The chapter identifies several highly similar phylotypes that co-occurred in fish feces, corals, and water. These co-occurrences

demonstrate the potential for bacteria to be transported from fish farms onto nearby corals.

Chapter 3 describes microbial assemblages in the guts and feces of three fish species collected at Palmyra Atoll. Cell abundances were high and varied by two orders of magnitude among the fishes. Feces bacteria were capable of growth and may have been associated with hydrolytic enzyme activity in the feces. Community composition was dominated by Vibrionaceae in two of the fishes, and phylogenetic analyses of 16S rRNA genes identified unique vibrio-like taxa.

Chapter 4 describes the potential for some bacterial phylotypes associated with parrotfish to persist in coral reefs at Palmyra Atoll.

Chapter 5 An appendix chapter describes how parrotfish feces can stimulate coral mucus production and the changes that microbial communities may experience in this process. It also presents estimates of parrotfish feces flux. The study was conducted at Palmyra Atoll concurrently with those described in Chapter 4.

Chapter 6 describes a new approach for observing DNA synthesis in growing marine bacteria. The thymidine analog ethynyldeoxuridine (EdU) becomes incorporated into DNA of growing cells and 'click' chemistry is used to label EdU with a fluorophore, which can be detecting via epifluorescence microscopy. The method was optimized in coastal seawater assemblages and may be applicable to determination of bacterial growth associated with particles including fish feces.

Chapter 7 states some conclusions and thoughts on potential future studies that expand on the ideas presented herein.

REFERENCES

Azam F & Malfatti F (2007) Microbial structuring of marine ecosystems. *Nature Reviews Microbiology* **5**: 782-791.

Bailey TG & Robertson DR (1982) Organic and Caloric Levels of Fish Feces Relative to its Consumption by Coprophagous Reef Fishes. *Marine Biology* **69**: 45-50.

Bellwood DR (1995) Direct estimate of bioerosion by two parrotfish species, Chlorurus gibbus and C. sordidus, on the Great Barrier Reef, Australia. *Marine Biology* **121**: 419-429.

Bidle KD & Azam F (1999) Accelerated dissolution of diatom silica by marine bacterial assemblages. *Nature* **397**: 508-512.

Bidle KD, Manganelli M & Azam F (2002) Regulation of oceanic silicon and carbon preservation by temperature control on bacteria. *Science* **298**: 1980-1984.

Fuhrman JA & Azam F (1982) Thymidine Incorporation as a Measure of Heterotrophic Bacterioplankton Production in Marine Surface Waters - Evaluation and Field Results. *Marine Biology* **66**: 109-120.

Geesey GG, Alexander GV, Bray RN & Miller AC (1984) Fish fecal pellets are a source of minerals for inshore reef communities. *Marine Ecology-Progress Series* **15**: 19-25.

Hamasaki K, Long RA & Azam F (2004) Individual cell growth rates of marine bacteria, measured by bromodeoxyuridine incorporation. *Aquatic Microbial Ecology* **35**: 217-227.

Jacobsen TR & Azam F (1984) Role of Bacteria in Copepod Fecal Pellet Decomposition - Colonization, Growth-Rates and Mineralization. *Bulletin of Marine Science* **35**: 495-502.

Lawrence SG, Ahmad A & Azam F (1993) Fate of Particle-Bound Bacteria Ingested by Calanus-Pacificus. *Marine Ecology-Progress Series* **97**: 299-307.

Porto I, Granados C, Restrepo JC & Sanchez JA (2008) Macroalgal-Associated Dinoflagellates Belonging to the Genus Symbiodinium in Caribbean Reefs. *Plos One* **3**: 5.

Robertson DR (1982) Fish Feces as Fish Food on a Pacific Coral Reef. *Marine Ecology-Progress Series* **7**: 253-265.

Smith DC, Simon M, Alldredge AL & Azam F (1992) Intense Hydrolytic Enzyme-Activity on Marine Aggregates and Implications for Rapid Particle Dissolution. *Nature* **359**: 139-142.

Verdugo P, Alldredge AL, Azam F, Kirchman DL, Passow U & Santschi PH (2004) The oceanic gel phase: a bridge in the DOM-POM continuum. *Marine Chemistry* **92**: 67-85.

CHAPTER 2: Gradients of coastal fish farm effluents and their effect on coral reef microbes

Environmental Microbiology (2008) **10**(9), 2299–2312 doi:10.1111/j.1462-2920.2008.01654.x

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

Melissa Garren,*† Steven Smriga† and Farooq Azam
Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, 8750 Biological Grade, Hubbs Hall, rm 4200, La Jolla, CA 92093-0202, USA.

Summary

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

Received 19 February, 2008; accepted 5 April, 2008. *For correspondence. E-mail mgarren@ucsd.edu; Tel. (+1) 858 534 3196; Fax (+1) 858 534 6478. †These authors contributed equally to this study.
Location of Study: Bolinao, Pangasinan, Republic of the Philippines.

Introduction

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

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

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

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

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

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

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

Results

Nutrients and chlorophyll a

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

Abundances of bacteria, particles and viruses

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

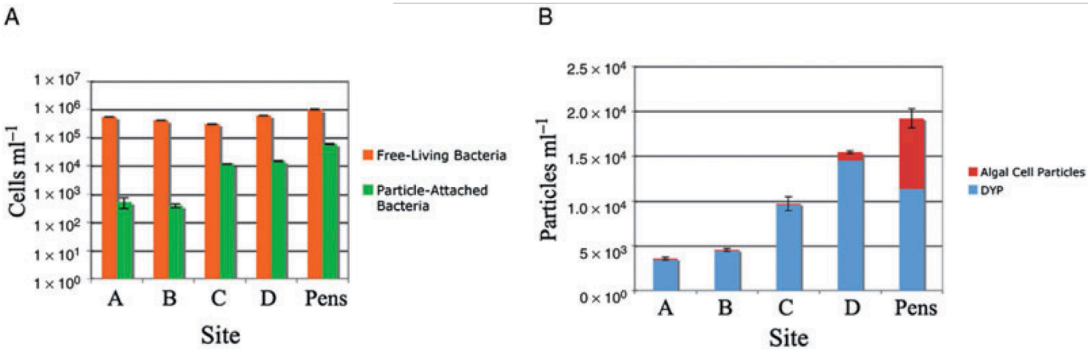


Fig. 1. A. Abundance of free-living and particle-attached bacteria in the water column.
B. Total particle abundance separated into the two categories of particles quantified: algal cell-derived and DAPI stained yellow particles (DYP). All error bars represent standard error. Data also presented in Table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

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

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

Microbial community composition

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

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

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

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

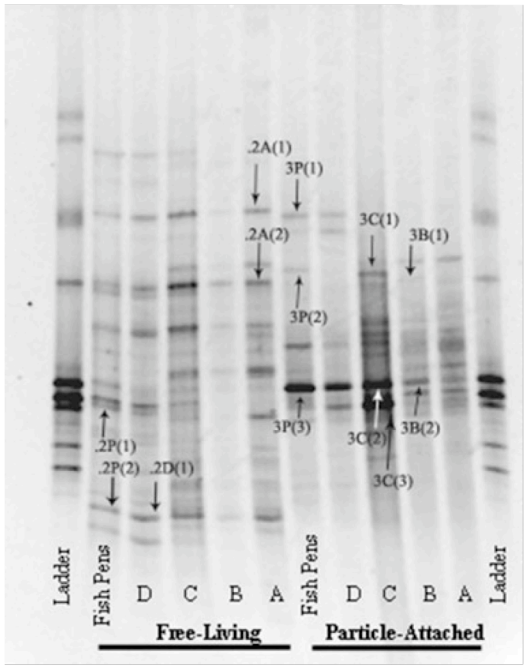


Fig. 2. DGGE banding profiles (V3 region of 16S rRNA gene) of water from each site revealed distinct free-living and particle-attached communities as well as a few phylotypes that appeared near the pens [e.g. 3P(1), 2P(2), 2D(1)]. Each lane contains pooled PCR products from triplicate water samples.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

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

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

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

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

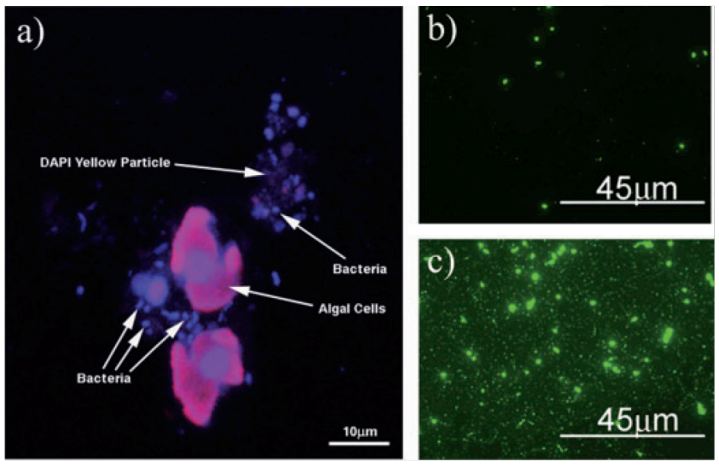


Fig. 3. Scanning laser confocal microscopy allows three-dimensional images to be taken of particles with attached bacteria. A representative maximum-projection confocal image of a DAPI-stained particle from the fish pen site (A) illustrates how bacteria can be enumerated on all sides of the particle.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

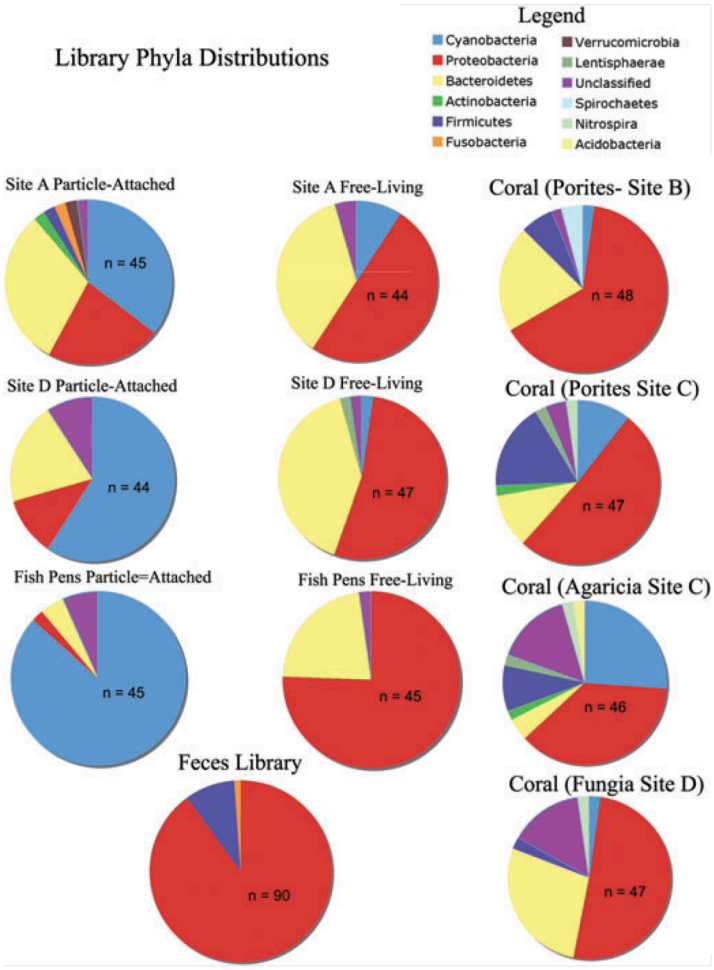


Fig. 4. The distribution of phyla in each 16S rRNA library (from particle-attached water, free-living water, coral tissue, and milkfish feces samples) where the number of sequences analysed for a single clone library is noted in the pie chart.

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

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

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

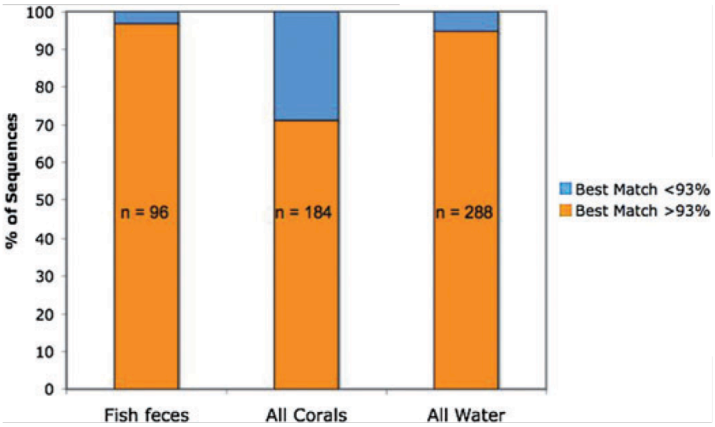


Fig. 5. Percent of novel sequences in 16S rRNA clone libraries. 'Novel' is defined as those that had < 93% best match similarity to the NCBI nt database. About 30% of sequences from coral samples were novel. The total number of sequences analysed for each sample type is presented.

Highly similar shared phylotypes among different sample types

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Experimental procedures

Study site and sampling locations

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

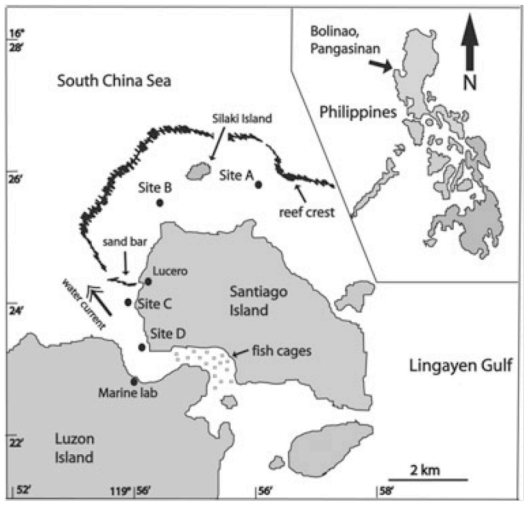


Fig. 6. Location of the study sites in Bolinao, Pangasinan, Republic of the Philippines.

Sample collection

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

Sample preservation for DNA

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

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

Chlorophyll

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

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

Dissolved and particulate organic carbon and nitrogen

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

Bacteria and particle abundances

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

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

PCR amplification of 16S rRNA gene sequences

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

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

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

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

Cloning

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

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

Denaturing gradient gel electrophoresis analyses

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

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

Sequence analyses

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

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

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

Statistical analyses

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

Acknowledgements

We thank all of the World Bank and Global Environment Facility Coral Disease Working Group members for their collaboration. We are especially grateful to our hosts at the University of the Philippines, Dr Edgardo Gomez, Dr Wolfgang Reichardt, Dr James Guest, Ms Marilou Martin, Mr Mark Arboleda, and Ms Miahnie Pueblos. Thanks to Drs Drew Harvell, Jessica Ward, Forest Rohwer and Eric Allen for helpful comments that greatly improved this manuscript. This research was supported by grants from the GEF Coral Targeted Research Program, Gordon and Betty Moore Foundation, and National Science Foundation (NSF) Grant No. 071463–002. Melissa Garren was supported by an NSF Integrative Graduate Education and Research Training (IGERT) Fellowship. Steven Smriga was supported by an NSF Graduate Research Fellowship.

References

Acinas, S.G., Sarma-Rupavtarm, R., Klepac-Ceraj, V., and Polz, M.F. (2005) PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. *Appl Environ Microbiol* **71:** 8966–8969.

Albright, L.J., McCrae, S.K., and May, B.E. (1986) Attached and free-floating bacterioplankton in Howe Sound, British-Columbia, a coastal marine fjord-embayment. *Appl Environ Microbiol* **51:** 614–621.

Azam, F., and Malfatti, F. (2007) Microbial structuring of marine ecosystems. *Nature Reviews Microbiology* **5:** 782–791.

Barneah, O., Ben-Dov, E., Kramarsky-Winter, E., and Kushmaro, A. (2007) Characterization of black band disease in Red Sea stony corals. *Environ Microbiol* **9:** 1995–2006.

Bascompte, J., Melian, C.J., and Sala, E. (2005) Interaction strength combinations and the overfishing of a marine food web. *Proc Natl Acad Sci USA* **102:** 5443–5447.

Bidle, K.D., and Fletcher, M. (1995) Comparison of free-living and particle-associated bacterial communities in the Chesapeake Bay by stable low-molecular-weight RNA analysis. *Appl Environ Microbiol* **61:** 944–952.

Bidle, K.D., and Azam, F. (1999) Accelerated dissolution of diatom silica by marine bacterial assemblages. *Nature* **397:** 508–512.

Brachvogel, T., Schweitzer, B., and Simon, M. (2001) Dynamics and bacterial colonization of microaggregates in a large mesotrophic lake. *Aquat Microbial Ecol* **26:** 23–35.

Buchan, A., Gonzalez, J.M., and Moran, M.A. (2005) Overview of the marine *Roseobacter lineage*. *Appl Environ Microbiol* **71:** 5665–5677.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., *et al.* (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Res* **35:** D169–D172.

Cottrell, M.T., and Kirchman, D.L. (2000) Natural assemblages of marine proteobacteria and members of the cytophaga-flavobacter cluster consuming low- and high-molecular-weight dissolved organic matter. *Appl Environ Microbiol* **66:** 1692–1697.

Crump, B.C., Baross, J.A., and Simenstad, C.A. (1998) Dominance of particle-attached bacteria in the Columbia River estuary, USA. *Aquat Microb Ecol* **14:** 7–18.

Davidson, K., Gilpin, L.C., Hart, M.C., Fouilland, E., Mitchell, E., Calleja, I.A., *et al.* (2007) The influence of the balance of inorganic and organic nitrogen on the trophic dynamics of microbial food webs. *Limnol Oceanogr* **52:** 2147–2163.

Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K., and Mattick, J.S. (1991) Touchdown PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res* **19:** 4008.

van Duyl, F.C., and Gast, G.J. (2001) Linkage of small-scale spatial variations in DOC, inorganic nutrients and bacterioplankton growth with different coral reef water types. *Aquat Microb Ecol* **24:** 17–26.

Enell, M. (1995) Environmental impact of nutrients from Nordic fish farming. *Water Sci Technology* **31:** 61–71.

Fabricius, K.E. (2005) Effects of terrestrial runoff on the ecology of corals and coral reefs: review and synthesis. *Mar Pollut Bull* **50:** 125–146.

Frias-Lopez, J., Zerkle, A.L., Bonheyo, G.T., and Fouke, B.W. (2002) Partitioning of bacterial communities between seawater and healthy, black band diseased, and dead coral surfaces. *Appl Environ Microbiol* **68:** 2214–2228.

Gast, G.J., Jonkers, P.J., van Duyl, F.C., and Bak, R.P.M. (1999) Bacteria, flagellates and nutrients in island fringing coral reef waters: influence of the ocean, the reef and eutrophication. *Bull Mar Sci* **65:** 523–538.

Gonzalez, J.M., Simo, R., Massana, R., Covert, J.S., Casamayor, E.O., Pedros-Alio, C., and Moran, M.A. (2000) Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom. *Appl Environ Microbiol* **66:** 4237–4246.

Grimes (1991) Ecology of estuarine bacteria capable of causing human disease: a review. *Estuaries* **14:** 345–360.

Grossart, H.P., and Simon, M. (1998) Bacterial colonization and microbial decomposition of limnetic organic aggregates (lake snow). *Aquat Microb Ecol* **15:** 127–140.

Hall, P.O.J., Anderson, L.G., Holby, O., Kollberg, S., and Samuelsson, M.O. (1990) Chemical fluxes and mass balances in a marine fish cage farm. 1. Carbon. *Mar Ecol Prog Ser* **61:** 61–73.

Hasegawa, T., Fukuda, H., and Koike, I. (2005) Effects of glutamate and glucose on N cycling and the marine plankton community. *Aquat Microb Ecol* **41:** 125–130.

Havskum, H., Thingstad, T.F., Scharek, R., Peters, F., Berdalet, E., Sala, M.M., *et al.* (2003) Silicate and labile DOC interfere in structuring the microbial food web via algal-bacterial competition for mineral nutrients: results of a mesocosm experiment. *Limnol Oceanogr* **48:** 129–140.

Holm-Hansen, O., Lorenzen, C.J., Holmes, R.W., Stickland, J.D.H. (1965) Fluorometric determination of chlorophyll. *J. Cons., Consperm Int Explor Mer* **30:** 3–15.

Holmer, M., and Kristensen, E. (1992) Impact of marine fish cage farming on metabolism and sulfate reduction of underlying sediments. *Mar Ecol Prog Ser* **80:** 191–201.

Holmer, M., Marba, N., Terrados, J., Duarte, C.M., and Fortes, M.D. (2002) Impacts of milkfish (*Chanos chanos*) aquaculture on carbon and nutrient fluxes in the Bolinao area, Philippines. *Mar Pollut Bull* **44:** 685–696.

Holmer, M., Duarte, C.M., Heilskov, A., Olesen, B., and Terrados, J. (2003) Biogeochemical conditions in sediments enriched by organic matter from net-pen fish farms in the Bolinao area, Philippines. *Mar Pollut Bull* **46:** 1470–1479.

Hughes, T.P., and Connell, J.H. (1999) Multiple stressors on coral reefs: a long-term perspective. *Limnol Oceanogr* **44:** 932–940.

Huq, A., Small, E.B., West, P.A., Huq, M.I., Rahman, R., and Colwell, R.R. (1983) Ecological relationships between *Vibrio-Cholerae* and planktonic crustacean copepods. *Appl Environ Microbiol* **45:** 275–283.

Joint, I., Henriksen, P., Fonnes, G.A., Bourne, D., Thingstad, T.F., and Riemann, B. (2002) Competition for inorganic nutrients between phytoplankton and bacterioplankton in nutrient manipulated mesocosms. *Aquat Microb Ecol* **29:** 145–159.

Karakassis, I., Tsapakis, M., Hatziyanni, E., Papadopoulou,

K.N., and Plaiti, W. (2000) Impact of cage farming of fish on the seabed in three Mediterranean coastal areas. *Ices J Mar Sci* **57:** 1462–1471.

Kirchman, D.L. (1994) The uptake of inorganic nutrients by heterotrophic bacteria. *Microb Ecol* **28:** 255–271.

Kirchman, D.L., Elifantz, H., Dittel, A.I., Malmstrom, R.R., and Cottrell, M.T. (2007) Standing stocks and activity of *Archaea* and *Bacteria* in the western Arctic Ocean. *Limnol Oceanogr* **52:** 495–507.

Kooperman, N., Ben-Dov, E., Kramarsky-Winter, E., Barak, Z., and Kushmaro, A. (2007) Coral mucus-associated bacterial communities from natural and aquarium environments. *Fems Microbiol Lett* **276:** 106–113.

Koren, O., and Rosenberg, E. (2006) Bacteria associated with mucus and tissues of the coral *Oculina patagonica* in summer and winter. *Appl Environ Microbiol* **72:** 5254–5259.

Long, R., and Azam, F. (2001) Microscale patchiness of bacterioplankton assemblage richness in seawater. *Aq. Mircob. Ecology* **26**:103–113.

Loya, Y., Lubinevsky, H., Rosenfeld, M., and Kramarsky-Winter, E. (2004) Nutrient enrichment caused by *in situ* fish farms at Eilat, Red Sea is detrimental to coral reproduction. *Mar Pollut Bull* **49:** 344–353.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**:1363–1371.

Mitchell, R., and Chet, I. (1975) Bacterial attack of coral in polluted seawater. *Microb Ecol* **2:** 227–233.

Moran, M.A., Gonzalez, J.M., and Kiene, R.P. (2003) Linking a bacterial taxon to sulfur cycling in the sea: studies of the marine *Roseobacter* group. *Geomicrobiol J* **20:** 375–388.

Morris, R.M., Longnecker, K., and Giovannoni, S.J. (2006) Pirellula and OM43 are among the dominant lineages identified in an Oregon coast diatom bloom. *Environ Microbiol* **8:** 1361–1370.

Mostajir, B., Dolan, J.R., and Rassoulzadegan, F. (1995) A simple method for the quantification of a class of labile marine pico- and nano-sized detritus: DAPI Yellow particles (DYP). *Aquat Microb Ecol* **9:** 259–266.

Mourino-Perez, R.R., Worden, A.Z., and Azam, F. (2003) Growth of *Vibrio cholerae* O1 in red tide waters off California. *Appl Environ Microbiol* **69:** 6923–6931.

Muyzer, G., Waal, E., and Uitterlinden, A. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**:695–700.

Nealson, K.H., and Venter, J.C. (2007) Metagenomics and the global ocean survey: what's in it for us, and why should we care? *Isme J* **1:** 185–187.

Pandolfi, J.M., Bradbury, R.H., Sala, E., Hughes, T.P., Bjorndal, K.A., Cooke, R.G., *et al.* (2003) Global trajectories of the long-term decline of coral reef ecosystems. *Science* **301:** 955–958.

Paul, J.H., Rose, J.B., Jiang, S.C., London, P., Xhou, X.T., and Kellogg, C. (1997) Coliphage and indigenous phage in Mamala Bay, Oahu, Hawaii. *Appl Environ Microbiol* **63:** 133–138.

Riemann, L., Steward, G.F., and Azam, F. (2000) Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Appl Environ Microbiol* **66:** 578–587.

Rohwer, F., Seguritan, V., Azam, F., Knowlton, N. (2002) Diversity and distribution of coral-associated bacteria. *MEPS* **243:** 1–10.

Sara, G. (2007) Ecological effects of aquaculture on living and non-living suspended fractions of the water column: a meta-analysis. *Water Res* **41:** 3187–3200.

Sekar, R., Mills, D.K., Remily, E.R., Voss, J.D., and Richardson, L.L. (2006) Microbial communities in the surface mucopolysaccharide layer and the black band microbial mat of black band-diseased *Siderastrea siderea*. *Appl Environ Microbiol* **72:** 5963–5973.

Simon, M., Grossart, H.P., Schweitzer, B., and Ploug, H. (2002) Microbial ecology of organic aggregates in aquatic ecosystems. *Aquat Microb Ecol* **28:** 175–211.

Thompson, F.L., Thompson, C.C., Naser, S., Hoste, B., Vandemeulebroecke, K., Munn, C., *et al.* (2005) *Photobacterium rosenbergii* sp. nov and *Enterovibrio coralii* sp. nov., vibrios associated with coral bleaching. *Int J Syst Evol Microbiol* **55:** 913–917.

Thompson, J.R., Marcelino, L.A., and Polz, M.F. (2002) Heteroduplexes in mixed template amplifications: formation, consequences, and elimination by 'reconditioning PCR'. *Nucleic Acid Rez* **30:** 2083–2088.

Torreton, J.P., and Dufour, P. (1996a) Temporal and spatial stability of bacterioplankton biomass and productivity in an atoll lagoon. *Aquat Microb Ecol* **11:** 251–261.

Torreton, J.P., and Dufour, P. (1996b) Bacterioplankton production determined by DNA synthesis, protein synthesis, and frequency of dividing cells Tuamotu atoll lagoons and surrounding ocean. *Microbial Ecology* **32:** 185–202.

Villanueva, R.D., Yap, H.T., and Montano, M.N.E. (2005) Survivorship of coral juveniles in a fish farm environment. *Mar Pollut Bull* **51:** 580–589.

Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73:** 5261–5267.

Wegley, L., Edwards, R., Rodriguez-Brito, B., Liu, H., and Rohwer, F. (2007) Metagenomic analysis of the microbial community associated with the coral *Porites astreoides* enable fixed nitrogen to cycle within the coral holobiont. *Environ Microbiol* **9:** 2707–2719.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic Study. *Journal of Bacteriology*, **173:** 697–703.

Worden, A.Z., Seidel, M., Smriga, S., Wick, A., Malfatti, F., Bartlett, D., and Azam, F. (2006) Trophic regulation of *Vibrio cholerae* in coastal marine waters. *Environ Microbiol* **8:** 21–29.

Chapter 2, in its entirety, is a reprint of the material as it appears in: Garren M, Smriga S, and Azam F (2008) Gradients of coastal fish farm effluents and their effect on coral reef microbes. *Environmental Microbiology* 10: 2299-2312. The dissertation author was one of two primary investigators and authors that contributed equally to the manuscript.

CHAPTER 3: Abundance, diversity, and activity of microbial assemblages associated with coral reef fish guts and feces

FEMS MICROBIOLOGY ECOLOGY

RESEARCH ARTICLE

Abundance, diversity, and activity of microbial assemblages associated with coral reef fish guts and feces

Steven Smriga, Stuart A. Sandin & Farooq Azam

Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, CA, USA

Correspondence: Steven Smriga, 9500 Gilman Dr., Mail Code 0202, La Jolla, CA 92093, USA. Tel.: +1 858 534 3196; fax: +1 858 534 7313; e-mail: ssmriga@ucsd.edu

Received 1 May 2009; revised 8 March 2010; accepted 9 March 2010.

DOI:10.1111/j.1574-6941.2010.00879.x

Editor: Julian Marchesi

Keywords
bacteria abundance; 16S rRNA gene phylogeny; hydrolytic enzymes; *Vibrio*; *Photobacterium*.

Abstract

Feces and distal gut contents were collected from three coral reef fish species. Bacteria cell abundances, as determined via epifluorescence microscopy, ranged two orders of magnitude among the fishes. Mass-specific and apparent cell-specific hydrolytic enzyme activities in feces from *Chlorurus sordidus* were very high, suggesting that endogenous fish enzymes were egested into feces. Denaturing gradient gel electrophoresis profiles of 16S rRNA genes were more similar among multiple individuals of the surgeonfish *Acanthurus nigricans* than among individuals of the parrotfish *C. sordidus* or the snapper *Lutjanus bohar*. Analyses of feces-derived 16S rRNA gene clones revealed that at least five bacterial phyla were present in *A. nigricans* and that *Vibrionaceae* comprised 10% of the clones. Meanwhile, *C. sordidus* contained at least five phyla and *L. bohar* three, but *Vibrionaceae* comprised 71% and 76% of the clones, respectively. Many sequences clustered phylogenetically to cultured *Vibrio* spp. and *Photobacterium* spp. including *Vibrio ponticus* and *Photobacterium damselae*. Other *Vibrionaceae*-like sequences comprised a distinct phylogenetic group that may represent the presence of 'feces-specific' bacteria. The observed differences among fishes may reflect native gut microbiota and/or bacterial assemblages associated with ingested prey.

Introduction

Microorganisms associated with particles in coastal marine ecosystems help drive biogeochemical cycles. For example, organic aggregates (marine snow) and phytoplankton detritus can be solubilized by attached bacteria expressing intense hydrolytic enzyme activity (Smith *et al.*, 1992; Bidle & Azam, 2001). Like other marine particles, animal feces are also subject to biogeochemical processes. Vertical or horizontal transport of feces in aquatic environments can create fluxes of organic matter that support biological processes (Wotton & Malmquist, 2001). Copepod fecal pellets, for example, can be colonized by bacteria at high concentrations and the colonizers experience rapid growth (Jacobsen & Azam, 1984; Lawrence *et al.*, 1993).

Unlike open ocean environments, where most sinking fecal pellets become dissolved within the water column and a minor fraction of fecal pellet organic matter settles to the bottom, coral reefs may experience a persistent 'rain' of feces. Because of the close physical proximity of fish to the benthos, fish feces may be an important mechanistic link between the pelagic food web and coral reefs. Feces from haemulids (grunts) that aggregate around coral colonies may provide nutrients and particulate organic carbon to corals and influence their growth rates; thus, fish feces may contribute to the health of live corals and other benthic organisms (Meyer & Schultz, 1985). Fish feces can also be consumed as food by fish, and coprophagy among fishes may be influenced by the caloric value of feces (Bailey & Robertson, 1982; Robertson, 1982).

Given the potential ecological roles for fish feces as particles in coral reefs, it is useful to consider the roles of fish gut- and feces-associated Bacteria and Archaea (henceforth called 'bacteria'). The abundances of culturable bacteria in the alimentary tracts of reef fishes can range from 10^6 to 10^9 g^{-1} wet wt (Sutton & Clements, 1988). Microbiota in some fish use fermentation to convert carbohydrates into short-chain fatty acids that can be absorbed by fish gut epithelial cells (Stevens & Hume, 1998; Clements & Raubenheimer, 2006). Proteins and vitamins produced by bacteria in the hindgut can also be absorbed by epithelial cells in some fishes (Stevens & Hume, 1998). With regard to

assemblage composition, culturable bacteria may be dominated by *Vibrio* spp. in some fishes (Sutton & Clements, 1988). Meanwhile, 'giant' *Epulopiscium* spp. have only been observed in surgeonfish guts, which suggests that some fish gut-associated bacterial phylotypes are specific symbionts (Angert *et al.*, 1993).

With some exceptions (Clements *et al.*, 2007), most previous studies of marine fish gut microbiota were culture dependent. Much remains to be known about the ecological roles of fish gut- and feces-associated bacteria, especially in healthy reef ecosystems. Palmyra Atoll in the central Pacific is encircled by nearly pristine reef ecosystems characterized by high live coral cover (Sandin *et al.*, 2008). Habitat-building herbivores such as parrotfish and top predators such as gray reef sharks and snappers are highly abundant and comprise $>50\%$ of the fish biomass (Sandin *et al.*, 2008). Given the low human impact on local fish populations, descriptive studies from Palmyra Atoll reflect a low likelihood of observing anthropogenic influences on feces-associated bacteria.

The following inter-related hypotheses were addressed using fish collected at Palmyra: (1) fish feces contain abundant microbial assemblages that have the potential to contribute to biogeochemical processes via hydrolytic enzyme activity and growth; (2) feces contain diverse microbial assemblages that vary among fish species; and (3) some bacterial phylotypes comprising distinct taxonomic groups are representative of fish feces. Three fishes with different diets representing two trophic levels were studied: *Chlorurus sordidus* (parrotfish) is a herbivore that consumes primarily endo- and epilithic algae; *Lutjanus bohar* (two-spot red snapper) is a top predator that consumes fishes and crustaceans; and *Acanthurus nigricans* (whitecheek surgeonfish) is a herbivore that consumes filamentous algae and detritus.

Materials and methods

Sample collection

Samples were collected from coral reef sites at Palmyra Atoll (5°53′N 162°05W) during August and September 2006. *Lutjanus bohar* were collected from outer reef sites using a line lure. For five individual fish, the mean weight ($\pm$ SD) was 1680 g ($\pm$ 1202), the mean standard length was 38.7 cm ($\pm$ 8.96), and the mean total length was 45.0 cm ($\pm$ 8.88). Gut contents from *L. bohar* were mucosal and highly viscous. Some feces were gray/black and some were beige/brown. The composition was not distinguishable. *Acanthurus nigricans* were collected at outer reef sites via SCUBA using a microspear. For six individuals, the mean weight ($\pm$ SD) was 110 g ($\pm$ 51.6), the mean standard length was 13.5 cm ($\pm$ 2.83), and the mean total length was 16.5 cm ($\pm$ 3.41). Gut contents from *A. nigricans* contained soft algal fragments encapsulated in mucosal matrices. Red, green, and brown macroalgal fragments ranged up to $\sim$3 mm in length. *Lutjanus bohar* and *A. nigricans* were dissected and contents from the distal colons were collected aseptically (henceforth called 'feces').

Feces from five *C. sordidus* (initial phase) were collected at backreef sites, but the fish were not harvested due to regulatory permitting constraints. Instead, fish were tracked via SCUBA until a defecation event occurred, and a sterile 5-mL syringe was used to collect the feces immediately after it had settled onto the substrate. One sample was collected each day over 5 days. Care was taken to minimize the collection of benthic substrate that might contaminate the feces sample. Feces from *C. sordidus* were granular and composed primarily of coral-derived carbonates and inorganic sediments. Small fragments of red and green algae were observed within the granular carbonate matrix. The composition appeared to be homogenous across the five individuals collected.

Aliquots of feces from all fish species were preserved in screw cap sterile tubes for C-H-N analyses, or in tubes containing RNAlater^{TM} (Ambion) for DNA analyses or 0.2 μm filter-sterilized 2% formaldehyde-seawater for microscopy. Preserved samples were stored immediately at $-20\,^{\circ}$C.

Feces composition and microbial abundances

Feces samples were acidified, and elemental C-H-N compositions were determined using an automated organic elemental analyzer (Analytical Laboratory, Scripps Institution of Oceanography, University of California, San Diego).

Microbial abundances were estimated using epifluorescence microscopy. Feces from five individuals per fish species were observed. Briefly, frozen aliquots of formaldehyde-fixed feces were thawed, amended with methanol (10% v/v), and sonicated to detach cells from feces particles (Lunau *et al.*, 2005). Postsonication samples were collected onto 0.2-μm pore-size polycarbonate filters (Millipore) and mounted onto glass slides with VectaShield containing 4′,6-diamidino-2-phenylindole (Invitrogen). To normalize by dry weight, aliquots of the fixed feces slurry were collected onto parallel membrane filters, dried to completion at 60 °C, and weighed via microbalance to determine the fecal mass per slurry volume. This weight and the cells per volume slurry were used to calculate cells g^{-1} dry wt. Percent water content was not determined.

Statistical analyses

The software package SIGMASTAT was used for statistical analyses. For carbon to nitrogen ratios, the Kruskal–Wallis ANOVA on ranks was performed. For microbial abundance data, normality and homogeneity of variance were demonstrated, and an ANOVA was performed to quantify the

effect of 'fish species' on microbial abundance. A *post hoc* least-squares means test with a Tukey's adjustment was performed for ANOVA results, where $P < 0.05$.

Enzyme activities

Enzyme activities on feces of individual *C. sordidus* were determined using the fluorogenic substrates leucine-7-amino-4-methylcoumarin (AMC) for aminopeptidases, 4-methylumbelliferyl (MUF)-phosphate for phosphatases, and MUF-β-D-glucoside for β-glucosidases (Hoppe, 1983). Feces were diluted in filter-sterilized seawater to ~1.2 mg dry wt feces mL^{-1}. Fluorogenic substrates were added to 100 nM and incubated at 22 °C in the dark over the course of the ~1-h assays. Samples were assayed in triplicate. To control for possible adsorption of substrate onto feces particles, duplicate feces aliquots were sterilized via microwave for 5 min before dilution into assays; the results for 'sterile' feces were subtracted from live assays. Fluorescence was determined using a fluorometer (Hoefer TKO-100) calibrated with AMC and MUF. Mass-specific rates (mmol substrate g^{-1} dry wt feces h^{-1}) were calculated, and apparent cell-specific rates (amol substrate per cell h^{-1}) were estimated using the mass-specific rates and the mean total cell abundances. Enzyme activities were not determined for *L. bohar* and *A. nigricans* due to time constraints associated with collecting these specimens.

PCR amplification of 16S rRNA genes

DNA was extracted from feces samples using the UltraClean™ Soil Kit (MoBio). To amplify assemblage 16S rRNA genes from feces samples for denaturing gradient gel electrophoresis (DGGE) analyses, the V3 region was targeted using a nested PCR approach. Samples first underwent a 15-cycle amplification (3 min at 94 °C; 1 min at 50 °C; 3 min at 72 °C) using Eubacteria-specific primer 27F and universal primer 1492R (Weisburg *et al.*, 1991). PCR products (2 μL) from this step were then used as a template for a modified touchdown PCR using V3-specific primers 341F with a GC clamp and primer 534R (Muyzer *et al.*, 1993; Garren *et al.*, 2008). An initial 94 °C denaturing step for 5 min was followed by 30 cycles of amplification (3-min denaturation at 94 °C; 1-min annealing starting at 65 °C for the first cycle and reduced 0.5 °C per cycle to 50 °C; and 3-min extension at 72 °C) and a final extension of 10 min at 72 °C. Touchdown PCR products were separated by electrophoresis on a 1.0% agarose gel for confirmation of < 200-bp product. Each 50-μL PCR reaction contained 2 U of Taq DNA polymerase (Eppendorf 5 Prime kit), 0.4 μM of each primer, and 250 μM dNTP final concentration.

To amplify total assemblage 16S rRNA genes for clone libraries, primers 27F and 1492R were used. Amplification conditions were similar to the first step of the nested PCR approach (3 min at 94 °C; 1 min at 50 °C; 3 min at 72 °C), except that 30 total cycles were used. The PCR products were concentrated via ethanol precipitation and then processed through a three-cycle regeneration step to reduce the likelihood of heteroduplex formation (Thompson *et al.*, 2002). Four microliters of the 10-μL regenerated PCR product was used as a template for clone library construction. To attempt to amplify archaeal 16S rRNA genes, primers 21F and 958R were tested twice using the amplification conditions described above, except with annealing temperatures of 52 °C or 55 °C, respectively.

Cloning and sequencing

To observe the specific phylotype composition, 16S rRNA gene clone libraries were assembled for one individual feces from each fish species. PCR products (~1460 bp length) were cloned using Invitrogen's pCR4-TOPO for Sequencing Kit with Top-10 chemically competent cells following the manufacturer's instructions. Clones were submitted to a commercial sequencing service (Agencourt Genomic Services, MA) that included plasmid preparation. To obtain partial sequences, primer 27F was used for one-direction gene reads, which were performed using BigDye Terminator v3.1 (Applied Biosystems), and sequences were delineated using a PRISM™ 3730xl DNA Analyzer (Applied Biosystems). The number of partial clone sequences analyzed was 44 for *C. sordidus*, 46 for *L. bohar*, and 48 for *A. nigricans*. Nearly full-length (> 1400 bp) sequences were attained for select clones that grouped within a putative novel taxonomic cluster. To do this, primers M13F/M13R were used to amplify plasmid inserts, and then the PCR products were purified and submitted for direct sequencing (GeneWiz Inc., La Jolla, CA) using primer 1492R.

DGGE analyses

To survey 16S rRNA gene diversity among feces from multiple individuals of a fish species, samples were compared by DGGE. Four to five individual feces from each fish species were observed. PCR products (< 200 bp) were separated by GC content using a hot-bath DGGE system (CBS Scientific DGGE-2000). Aliquots of PCR products were loaded onto 8.0% polyacrylamide gels in 0.5 × TAE buffer (20 mM Tris, 10 mM sodium acetate, 0.5 mM Na_2EDTA, pH 8.2) with top-to-bottom denaturing gradients of 30–60% formamide and urea (100% denaturant being 40% v/v formamide and 7 M urea). Electrophoresis was run at 80 V for 16 h at 60 °C in 0.5 × TAE. After electrophoresis, the gels were stained for 30 min in 0.5 × SYBR Gold (Invitrogen) in TAE buffer. Gels were then imaged using a UVP Epi-chemi Darkroom with a charge-coupled device camera. Image exposure times were adjusted depending on light saturation for the most intense band.

Densitometry was used (IMAGEJ 1.39) to produce band intensity profiles for each sample from DGGE gel images (not shown). The Shannon–Weiner index (H') for each profile was determined using the following equation:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

where S is the number of bands in a profile (i.e. assemblage phylotype richness) and p_i the relative abundance of a band (i.e. phylotype), calculated as the intensity of the band divided by the total intensity of all bands in the profile (i.e. assemblage). The mean H' among samples is reported for each species. To estimate interindividual similarity within a fish species (i.e. pairwise band-based comparison of profiles within a fish species), Bray–Curtis similarity scores (BC_{ij}) were calculated using the following equation:

$$BC_{ij} = \sum \frac{|n_{ik} - n_{jk}|}{(n_{ik} + n_{jk})}$$

where BC_{ij} is the similarity between two samples i and j, and n_{ik} and n_{jk} the presence (1) or absence (0) of each DGGE band for i and j, respectively. The mean BC_{ij} among samples is reported as the similarity score for each fish species. Bands that appeared in feces profiles at the same location as bands in negative control amplifications were not included in the comparisons. Samples from the three fishes were run on two separate gels, but the gels did not include universal standards that allow accurate rescaling and normalization of banding profiles for comparison between the gels (Ferrari & Hollibaugh, 1999). Therefore, similarities between feces type (e.g. dendograms) were not performed, even while diversity indices and similarity comparisons among multiple samples for any one feces type are valid. Similarity and diversity calculations were based on the assumption that each single DGGE band represented a unique phylotype. However, it has been observed that some bacterial isolates yield multiple DGGE bands most likely due to PCR biases or multiple 16S rRNA gene loci within genomes (Crosby & Criddle, 2003). In an attempt to relate DGGE gels to clone libraries, a selection of DGGE bands were excised from gels and eluted into saline-sodium citrate buffer. A subset of the eluted products were PCR reamplified and run on a new DGGE gel to check the location of the product migration. Select products were cloned using the Invitrogen pCR4-TOPO kit and eight clones from each library were selected for comparison of the ~200 bp sequence. It was expected that all eight clones from any one library would match identically. However, we found that only two to three clones within any set of eight were 100% similar, while the remaining were 'singletons' that did not match any other. This may be explained by the comigration of different PCR products in the DGGE gel and/or reamplification and subsequent cloning of contaminating DNA or heteroduplexes from the excision/elution steps. Given these ambiguous results, phylogenetic identifications from excised bands of interest were excluded from this study.

Sequence analyses

Sequence trace files were imported and trimmed using the Ribosome Database Project (RDP-X) online portal (Wang *et al.*, 2007). Phyla designations were determined using the RDP-X Pipeline alignment and classification tools. Estimates for Chao and Ace richness were performed using DOTUR (Schloss & Handelsman, 2005) and estimates for homologous coverage were calculated using the formula $C_x = 1 - (N_x/n)$, where N_x is the number of unique sequences in the sample at the 97% similarity level and n is the total number of sequences.

To reconstruct phylogenies, trimmed sequences were aligned using the SINA WEBALIGNER (http://www.arb-silva.de/aligner/) and imported into SILVA SSUParc database version 92 via ARB (Pruesse *et al.*, 2007). For fish feces sequences that were $>$ 99% similar, the longest sequence in each consensus group was selected as a representative. Consensus sequences were detected with Chimera Detection and excluded from phylogenetic trees. Closely related aligned sequences were exported using the 'gamma_1_rr5_dec04' filter into PHYLIP 3.6 (Felsenstein, 2005), and PHYLIP Seqboot was used to produce 100 resamplings for each data set. Phylogenetic relationships were inferred from the Seqboot outfiles using neighbor joining, maximum parsimony, and maximum likelihood (ML). The tree results for each of the three methods were compiled using PHYLIP Consensus, the three outtrees were compiled into a final consensus tree, and branch lengths were assigned to the final tree via ML. Most *Vibrio* spp. reference sequences were selected based on the results of a previous taxonomic study (Thompson *et al.*, 2004). Some reference sequences were selected based on high similarity and relevant isolation source (e.g. diseased fishes). Sequences from a study of farmed milkfish in close proximity to coral reefs in the Philippines (Garren *et al.*, 2008) were included in the phylogenetic analyses.

All 16S rRNA gene sequences derived from this study were submitted to the GenBank database. Accession numbers are FJ653927–FJ653974 for sequences derived from *A. nigricans*, FJ653975–FJ654018 for *C. sordidus*, FJ654019–FJ654062 for *L. bohar*, and FJ654063–FJ654065 for milkfish.

Results

Feces bacterial abundances and composition

The mean total bacterial abundances g^{-1} dry wt feces ($\pm$ SE) were 1.71×10^9 ($\pm 7.21 \times 10^8$) for *C. sordidus*, 1.99×10^{10} ($\pm 8.29 \times 10^9$) for *L. bohar*, and 2.59×10^{11} ($\pm 3.11 \times 10^{10}$)

for *A. nigricans*. The total microbial abundances were significantly different among the three fish species ($F = 54.385$; $P < 0.001$; $n = 5$). The mean ratios of carbon to nitrogen (± SE) were 10.1 (± 0.271) for *C. sordidus*, 3.39 (± 0.633) for *L. bohar*, and 5.66 (± 1.87) for *A. nigricans*. The C : N ratios were significantly different ($H = 8.538$; $P = 0.002$).

Production rates for *C. sordidus* feces-associated bacteria

During incubations of *C. sordidus* feces, the mean total bacterial abundances (± SE) among five individual feces increased twofold from 1.71×10^9 g^{-1} dry wt feces ($\pm 7.21 \times 10^8$) at $t_{initial}$ to 3.59×10^9 g^{-1} dry wt feces ($\pm 9.07 \times 10^8$) at t_{final} (Table 1). This represents a mean production rate of 1.88×10^8 ($\pm 5.48 \times 10^7$) new bacteria g^{-1} dry wt feces h^{-1} and a mean generation time of 10.1 ± 2.8 h (Table 1). After incubation, cells in the assemblage appeared to be larger and more homogenous in morphology relative to $t_{initial}$.

Enzyme activity for *C. sordidus* feces

During incubations of *C. sordidus* feces, mass-specific aminopeptidase activity increased by 38% from $t_{initial}$ to t_{final} (Table 1), although the increase was not significant. Conversely, apparent cell-specific aminopeptidase activity decreased > 50% during the incubation (not shown), although the difference was also not significant. A similar pattern was observed for β-glucosidase activity, whereby mass-specific activity increased by 31% (Table 1), but apparent cell-specific activity decreased by 59%. Meanwhile, phosphatase activity followed a different pattern in that both mass-specific and cell-specific activities decreased by 17% and 75%, respectively. Like aminopeptidase, the differences between timepoints were not significant for either β-glucosidase or phosphatase activities.

DGGE profiles of bacterial assemblages

Acanthurus nigricans feces hosted higher bacterial phylotype richness than the other fish species when compared via DGGE. The mean number of DGGE bands for *A. nigricans* was significantly higher ($F = 11.496$; $P < 0.01$) and the Shannon–Weiner diversity index (H') for *A. nigricans* was moderately higher ($F = 3.304$; $P = 0.075$) than the other two fishes (Fig. 1). Additionally, the Bray–Curtis similarity score was the highest in *A. nigricans* feces (Fig. 1), indicating less variation among different individuals of this species compared with the other two species.

Clone library assessment of bacterial diversity

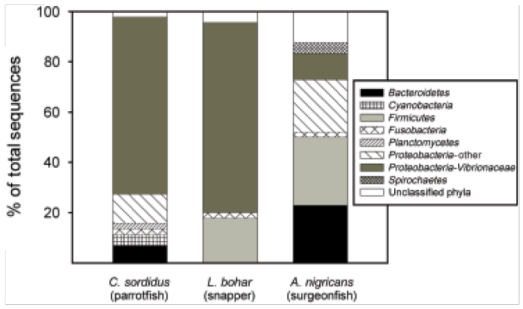
The number of bacterial phyla represented in 16S rRNA gene clone libraries was three for *L. bohar* and five for both *C. sordidus* and *A. nigricans* (Fig. 1). *Proteobacteria* represented the largest portion of the total classifiable sequences in all three fish species, although the portion was smaller in *A. nigricans* relative to the other fishes (Fig. 1). The percentage of the total library classified as *Vibrionaceae* (*Gammaproteobacteria*) was 75.6% in *L. bohar*, 70.5% in *C. sordidus*, and 10.4% for *A. nigricans*. Percent library coverage was the lowest, and estimators of phylotype richness (Chao-1 and Ace) were the highest, in *A. nigricans* feces (Fig. 1). The portion of each library that could not be classified within phyla was 12.5% for *A. nigricans*, while it was < 5.0% for both *C. sordidus* and *L. bohar* (Fig. 1). PCR amplification attempts using Archaea-specific primers produced no products from any of the three feces types. However, an amplification product was observed using DNA from archaeal isolate *Halobacterium salinarum* as a positive control.

Because 51% of the 138 total 16S rRNA gene sequences from the fishes were *Vibrionaceae*, and because clone libraries from cultured milkfish also contained several *Vibrionaceae* sequences (Garren *et al.*, 2008), detailed phylogenies were reconstructed for *Vibrionaceae*-like sequences. Twenty unique partial 16S rRNA genes (~650 bp covering hypervariable regions V1 through V3) representing 53 clones could be phylogenetically grouped within genus *Vibrio* (Fig. 2a). Many clone sequences clustered with representative strains of *Vibrio ponticus*, *Vibrio fortis*, *Vibrio qinhuangdaora*, and *Vibrio nigripulchritudo* (Fig. 2a). Other sequences clustered with *Vibrio agarivorans*, *Vibrio coralliilyticus*, and *Vibrio furnissii* (Fig. 2a). These latter three

Table 1. Bacteria abundances and mass-specific hydrolytic enzyme rates associated with *Chlorurus sordidus* feces incubated in filtered seawater

	$t_{initial}$	t_{final}	Incubation time (h)
Bacteria abundance (10^9 g^{-1} dry wt feces)	1.71 ± 0.721	3.59 ± 0.908	9.6 ± 0.35
Aminopeptidase	38.0 ± 16.9	52.6 ± 30.6	9.8 ± 0.41
Phosphatase	32.4 ± 20.7	26.8 ± 13.4	9.8 ± 0.41
β-Glucosidase	3.2 ± 1.1	4.2 ± 2.1	9.8 ± 0.41

Aminopeptidase rates are mmol AMC g^{-1} dry wt feces h^{-1}, while phosphatase and β-glucosidase rates are mmol MUF g^{-1} dry feces h^{-1}. Mean values (± SE) among five individual feces (collected on five different dates) are shown. For all values, $t_{initial}$ was ~3-h postegestion. For enzyme assays, analytical triplicates were used to determine a mean value for each individual feces (not shown). For bacteria abundances, the calculated production rate between $t_{initial}$ and t_{final} was $1.88 \pm 1.23 \times 10^8$ g^{-1} dry wt feces h^{-1} while the generation time was 10.1 ± 2.8 h.



		C. sordidus	*L. bohar*	*A. nigricans*
Clone Libraries	Total sequences	44	46	48
	% coverage	52.2	71.1	33.3
	Chao-1	32	18	139
	Ace	41	20	71
DGGE	# bands	16 (±2.3)	15 (±2.4)	30 (±2.5)
	Shannon-Weiner (*H'*)	2.06 (±0.20)	2.21 (±0.13)	2.65 (±0.15)
	Bray-Curtis similarity	58%	62%	78%

Fig. 1. Clone library and DGGE results for 16S rRNA genes from fish feces of *Chlorurus sordidus* and distal gut contents of *Lutjanus bohar* and *Acanthurus nigricans*. The clone library phyla distributions (bar chart) are for partial 16S rRNA genes (~600 bp, covering variable regions V1 through V3). The percent library coverage and Chao-1 and Ace estimators of phylotype richness for the clone libraries (top half of table) are all at the level of 97% sequence similarity. DGGE results (bottom half of table) show the mean values (± SE) among five individuals sampled per fish species.

'clusters' were less closely related to their nearest representative sequences than the *V. ponticus*-, *V. qinhuangdaora*-, and *V. nigripulchritudo*-associated clusters. In addition to genus *Vibrio*, five unique feces sequences representing 28 clones grouped within genus *Photobacterium* (Fig. 2b). Some sequences from *L. bohar* and Philippines milkfish feces clustered with *Photobacterium damselae*, while other sequences from *C. sordidus* clustered with *Photobacterium rosenbergii*.

Several *Vibrio* spp. and *Photobacterium* spp. 16S rRNA gene clone sequences were redundant (i.e. ≥99% aligned sequence similarity). There were 12 ≥99% consensus similarity sets among the *Vibrio* spp. sequences (Fig. 2a) and two among the *Photobacterium* spp. sequences (Fig. 2b).

While many sequences were *Vibrio* spp. and *Photobacterium* spp., a distinct cluster of seven sequences may represent a unique taxonomic group (Fig. 3). The cluster is comprised of two subgroups: one contains sequences from *A. nigricans* feces and the other contains sequences from *C. sordidus* feces. The sequences, which are > 1400 bp (see Materials and methods), grouped more closely to *Vibrionaceae* than to *Alteromonadaceae* or *Aeromonadaceae* (Fig. 3). The '*Enterovibrionaceae*' [represented by *Enterovibrio coralii* (accession no. AJ842343)] and '*Salinovibrionaceae*' (represented by *Salinivibrio costicola*) (Thompson *et al.*, 2004) were the most closely related *Vibrionaceae* representatives. However, the least-derived sequence in the cluster (AC.NIGR.02_clG10) was only 95.0% similar to *E. coralii* and 94.5% similar to *Grimontia hollisae* (AJ514909). Also, the highest similarity between any sequences of the two subgroups was 96.0% (CH.SORD.03_clB05 and AC.NIGR.02_clE12; data not shown). Therefore, the cluster, and particularly the *C. sordidus*-associated subgroup (Fig. 3), may represent an undescribed order within the class *Gammaproteobacteria* or an undescribed genus within the order *Vibrionaceae*. These nearly full-length sequences did not appear to contain chimeric or heteroduplex properties (see Materials and methods).

The *A. nigricans* feces clone library contained few *Vibrionaceae*-like sequences. However, among the diverse non-vibrio-like sequences were six that grouped phylogenetically to the 'giant' bacterium *Epulopiscium fishelsoni* (Fishelson *et al.*, 1985; Angert *et al.*, 1993). Two of the ~600 bp *A. nigricans* sequences (AC.NIGR_clG11 and AC.NIGR_clF09) were 95.0% and 95.2% similar to *E. fishelsoni* (M99574), respectively. The other four sequences were 95–96% similar to *Clostridiales* sequences from feces of various mammals (Ley *et al.*, 2008).

Discussion

Feces from these three fish species supported high bacterial cell abundances (10^9–10^{11} g^{-1} dry wt feces) that are comparable to results from previous studies of marine fish gut microbiota. In the cecal pouches of two subtropical herbivorous species (*Kyphosus* spp.), the abundances of culturable bacteria ranged from 2.8 to 5.0×10^{11} g^{-1} dry wt (Rimmer & Wiebe, 1987; Clements, 1997). In the hindguts of three Great Barrier Reef species – a planktivore (*Caesio erythrogaster*), a detritivore (*Ctenochactus striatus*), and a herbivore closely related to *A. nigricans* (*Acanthurus nigrofuscus*) – the abundances of culturable bacteria ranged from ~10^6 to ~10^9 g^{-1} hindgut content (Sutton & Clements, 1988). Given such high abundances, even rare bacterial phylotypes will be highly abundant. For example, while *Fusobacteria* comprised only about one percent of the phyla in each of the feces types (Fig. 1), this may reflect the presence of ~10^9 *Fusobacteria* cells g^{-1} dry wt feces.

In summarizing other studies, Clements (1997) noted that parrotfishes (family *Scaridae*) did not host an 'obvious microbiota.' The author suggested that parrotfish diets, which contain high proportions of inorganic sediments, create conditions that are unfavorable for robust microbial assemblages. Indeed, the C : N ratios observed for the three feces types (see Results) were consistent with the known diets for these three fish species. The parrotfish *C. sordidus* most likely scraped and ingested coral skeletons, while the

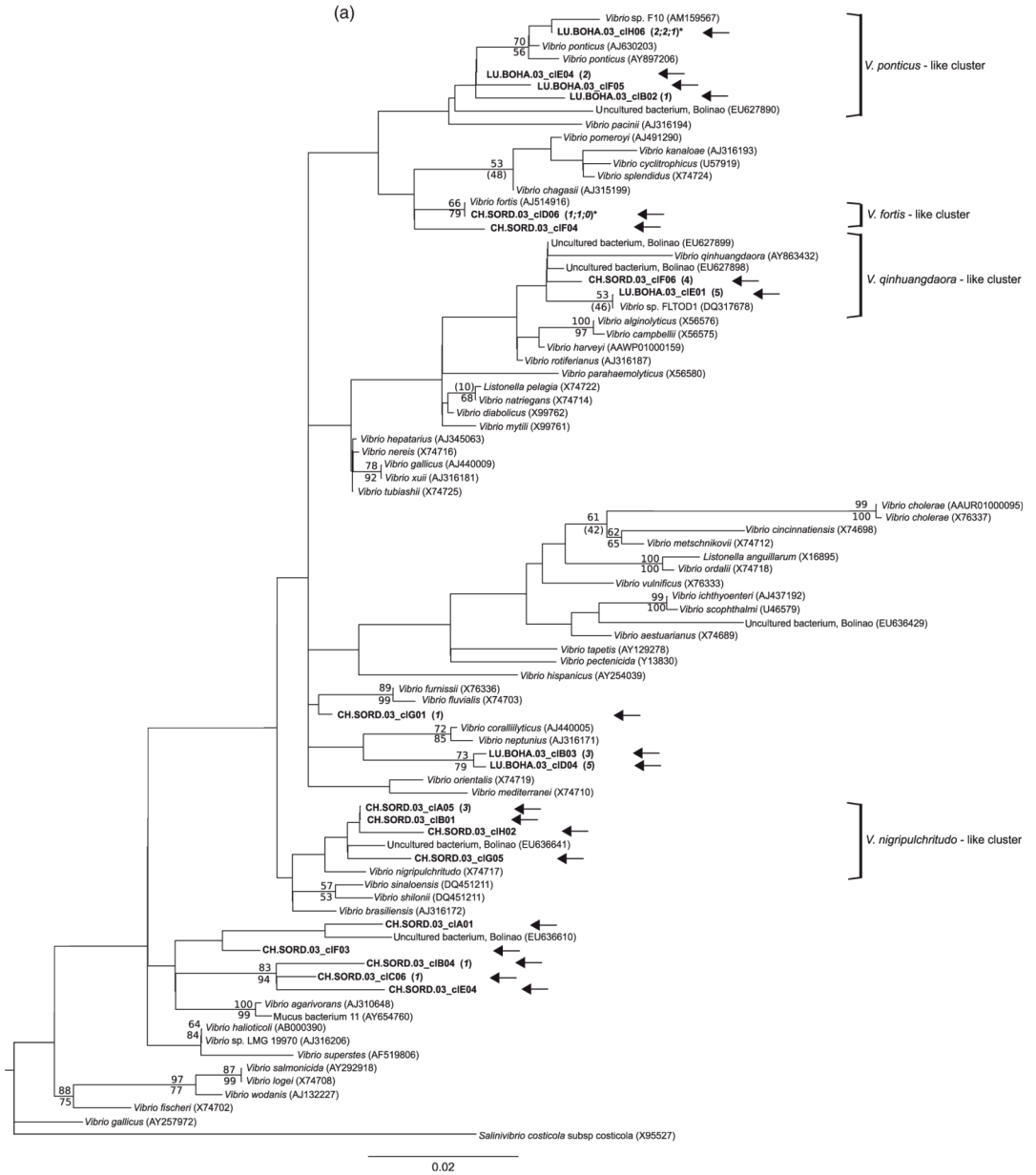


Fig. 2. ML-based consensus trees showing the phylogenetic relationships of 16S rRNA genes for (a) *Vibrio* spp. and (b) *Photobacterium* spp. from coral reef fish feces and distal gut contents (bold with arrows). The sequence length was partial (~650 bp covering variable regions V1 through V3) for fish feces/gut sequences and nearly complete (> 1400 bp) for reference species. Sequences from *Lutjanus bohar* gut contents contain the label 'LU.BOHA.03,' while those from *Chlorurus sordidus* feces contain the label 'CH.SORD.03'. Clone sequences from a previous study of farmed milkfish (Garren *et al.*, 2008) are denoted with 'Bolinao'. GenBank accession numbers for reference species are shown in parentheses. Parsimony bootstrap values (%) are indicated above branch nodes, while ML-bootstrap values (%) are indicated below. Only bootstrap values > 50% are shown (bootstrap values in parentheses were < 50%, but are shown for comparison). The scale bars represent (a) 2.0% and (b) 1.0% estimated evolutionary distance. Italicized numbers next to some sequences indicate the number of clones from the same library that were > 99% similar based on distance matrix consensus. Two *Vibrio* sequences indicated by (*) contained > 99% similar sequences from multiple sources; the first number indicates the same fish species, the second number indicates another species from this study, and the third number indicates the previous study of farmed milkfish.

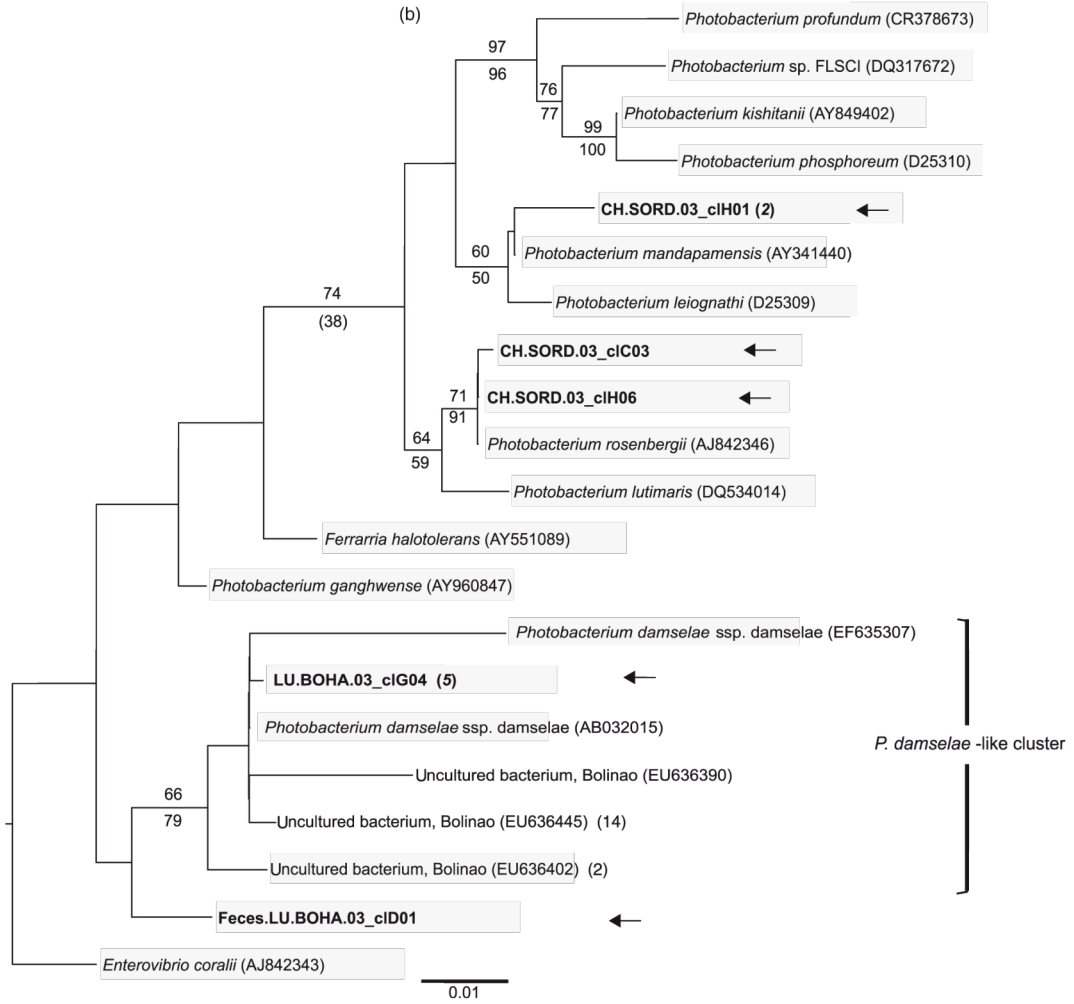


Fig. 2. Continued.

snapper *L. bohar* most likely ingested protein-rich fish prey. If C : N ratios reflect the capacity to support microbial biomass, then this is consistent with *C. sordidus* hosting the lowest bacterial abundances and *L. bohar* the highest. Despite these relative differences, *C. sordidus* feces contained $\sim 10^9$ bacteria g^{-1} dry wt, and given that the approximate dry weight of an individual parrotfish feces is 30 mg (data not presented), one egested pellet from *C. sordidus* contains at least 3×10^7 bacteria. Moreover, bacteria abundances in parrotfish feces increased during incubations (Table 1), and the increase was also observed qualitatively among feces particles that were examined by confocal laser scanning microscopy without sonication to separate attached bacteria (data not presented). This suggests that parrotfish feces-associated bacterial assemblages can experience rapid growth in the environment after egestion from the fish. We note that we cannot exclude the possibility that growth of bacteria resulted from experimental perturbation, whereby leaching of dissolved organic matter (DOM) from feces created organically rich conditions that promoted rapid bacterial growth.

Hydrolytic enzyme activity in feces from parrotfish and other species may enable solubilization of fecal particulate organic matter (POM) and therefore provide an important biogeochemical function in reef water. Mass-specific rates (i.e. bulk rates) describe the activities in total feces and are

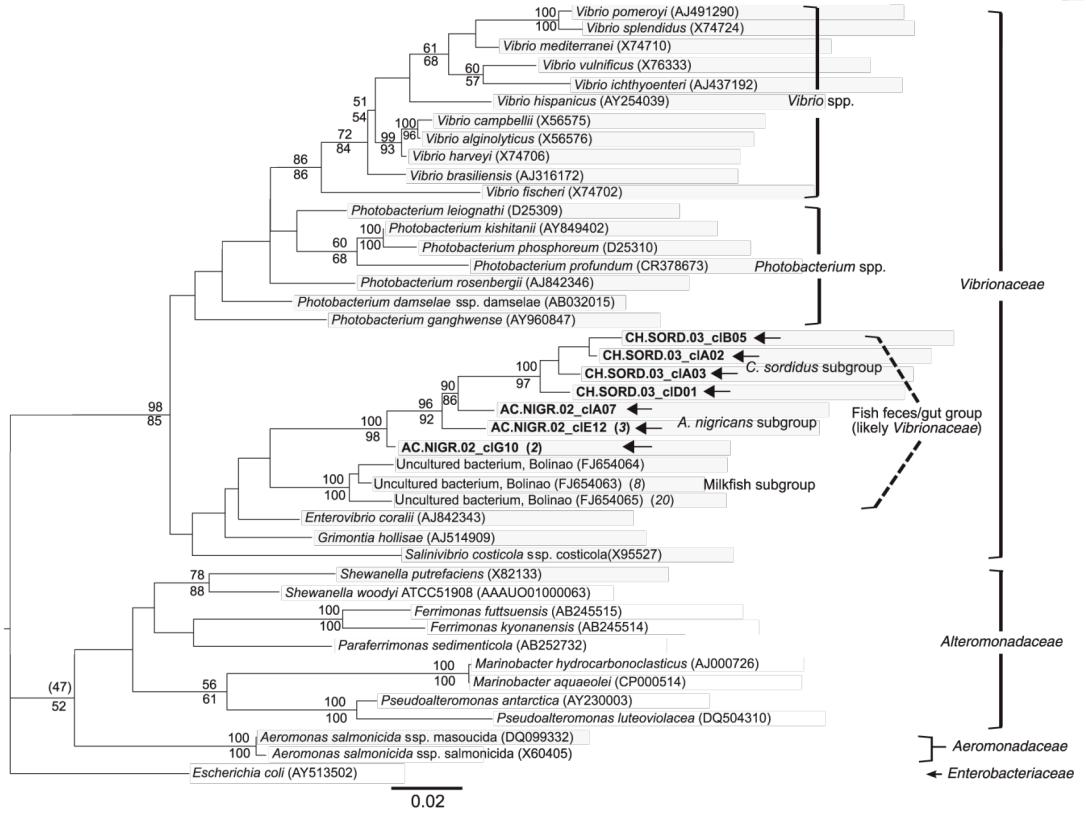


Fig. 3. A ML-based consensus tree showing the phylogenetic relationships of 16S rRNA gene sequences from coral reef fish feces and gut contents (bold with arrows). The sequence length was nearly complete (> 1400 bp) for both the reference species and the fish feces/gut sequences. Sequences from *Chlorurus sordidus* feces are labeled 'CH.SORD.03', those from *Acanthurus nigricans* gut contents are labeled 'AC.NIGR.02', and those from a previous study of farmed milkfish (Garren *et al.*, 2008) are denoted with 'Bolinao'. Parsimony bootstrap values (%) are indicated above branch nodes, while ML-bootstrap values (%) are indicated below. Only bootstrap values > 50% are shown (bootstrap values in parentheses were < 50%, but are shown for comparison). The scale bar represents the 2.0% estimated evolutionary distance. Italicized numbers next to some sequences indicate the number of clones from the same library that were > 99% similar based on distance matrix consensus.

different from cell-specific rates, which are calculated using the total bacteria abundance in a sample. Using the observed aminopeptidase activity in parrotfish feces (Table 1), we calculated a hydrolysis rate of 34–340 mmol AMC g^{-1} C h^{-1} (given percent organic carbon of feces range of 5–50%). This rate can be compared with previous observations of planktonic marine aggregates (Smith *et al.*, 1992), for which we calculated an aminopeptidase rate of ~700 mmol AMC g^{-1} C h^{-1}. Thus, the carbon mass-specific rates for marine aggregates and for parrotfish feces are within an order of magnitude of each other. Like marine aggregates in pelagic waters, egested parrotfish feces may be potential 'hotspots' of hydrolytic enzyme activity in coral reef waters. Fecal enzymatic hotspots may dissipate quickly if the enzymes are soluble in seawater.

In contrast to bulk rates, the calculated apparent cell-specific aminopeptidase activities from parrotfish feces (e.g. 26.3 fmol AMC per cell h^{-1}) were considerably higher than the cell-specific aminopeptidase activities previously estimated in planktonic marine aggregates [242 amol AMC per cell h^{-1}; (Smith *et al.*, 1992)]. Endogenous fish gut enzymes likely contributed considerably to enzymatic activity in the egested parrotfish feces. Indeed, endoproteases, peptidases, lipases, and carbohydrases are known to occur in fish gastrointestinal tracts (Clements & Raubenheimer, 2006). Some gut enzymes are produced by fish within the lumen membrane, while others may be produced by bacterial symbionts. Gut enzymes from either source could become entrained into feces upon egestion. Regardless of whether the source is endogenous or bacterial, feces-associated

enzymes likely provide an important mechanism for ecosystem-scale transition of fecal POM into DOM.

Analyses of 16S rRNA gene clone libraries revealed three distinguishable community composition patterns. First, feces from *C. sordidus* and *L. bohar* were dominated by phylotypes closely related to cultured, systematically described *Vibrio* and *Photobacterium* spp. (Fig. 2a and b). Several clones clustered with *V. ponticus*, a species originally isolated from diseased sea bream (Macian *et al.*, 2004) and a potential pathogen for Japanese sea bass (Xie *et al.*, 2007). Other clones clustered with *V. qinhuangdaora* [originally isolated from diseased flounder (Fang *et al.*, 2005)], *P. damselae* (a well-described fish pathogen), and *Photobacterium leiognathi* [a bioluminescent 'species' originally isolated from the light organs of ponyfish (Dunlap & Steinman, 1986)]. Given the sources of these described species, the clustering patterns (Fig. 2a and b) suggest that feces contained at least some bacterial phylotypes that occur commonly in marine fishes. The high proportion of *Vibrio* spp. clones in *C. sordidus* and *L. bohar* feces is concordant with prior analyses that demonstrated the dominance of vibrios among cultured isolates from three Great Barrier Reef fishes (Sutton & Clements, 1988).

The second pattern that emerged from clone libraries was the presence of a distinct phylogenetic cluster that may represent a novel taxonomic group (Fig. 3). The clade may be fish feces specific because no database sequences fell within the cluster. Because sequences in the cluster originated from two different fish species at Palmyra as well as in cultured milkfish in the Philippines, bacteria in this novel group may exist among diverse fish taxa and have a wide distribution in the Pacific.

A third discernible pattern was that the clone library from feces of the surgeonfish *A. nigricans* was not dominated by *Vibrio* spp. Instead, it contained numerous non-vibrio *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* sequences as well as representatives from other phyla. Indeed, estimators for phylotype richness were higher and the mean number of DGGE bands was nearly twofold greater relative to the other two fishes (Fig. 1), suggesting that microbial assemblage diversity was higher in *A. nigricans* feces. This is consistent with a previous study that found that cultured isolates from feces of *A. nigrofuscus* were dominated by agar-digesting non-vibrio bacteria and comprised communities that were distinct from three other Great Barrier Reef fishes (Sutton & Clements, 1988). Thus, *Acanthurus* spp. at two distinct reef ecosystems hosted bacterial assemblages that were more diverse than those found in other fish species.

The culture-independent phylogenetic analyses enabled classification of *Vibrionaceae* sequences and identification of uncultured phylotypes. We note that individual *Vibrio* genomes can contain up to 12 16S rRNA gene operons with $\leq$ 1.5% sequence variability, and such intragenomic heterogeneity can influence the diversity indices calculated from the DGGE profiles (Jensen *et al.*, 2009). However, it is unlikely to have accounted for the taxonomic clade identified in the fish feces clone library analyses (Fig. 3) because these sequences were $>$ 5% dissimilar from the nearest representative strain, *E. coralii*. While diverse Bacteria 16S rRNA genes were detected, amplification using Archaea-specific primers did not yield a PCR product. Given that archaea are abundant in marine waters, we cannot rule out that they were present in the fish feces, perhaps at a low abundance, but the complex sample matrices did not allow PCR amplification with Archaea primers. Further, a goal for the future is to determine the source of bacteria into the feces, for example the fish intestine, stomach, ingested prey (including prey feces), or free-living in seawater.

Conclusions

High microbial abundances in coral reef fish feces ranging from 10^9 to 10^{11} g^{-1} dry wt feces may drive biogeochemical processes via hydrolytic enzyme activity and through conversion of organic matter into bacterial biomass during growth. Unique taxonomic groups of bacteria found in the environment, such as that identified in *C. sordidus* and *A. nigricans* feces, may represent episodic or persistent interactions with feces from specific fish. The results can be considered within the ecological context of the coral reefs at Palmyra Atoll. Functionally intact fish assemblages such as those at this remote and unfished atoll enable tight coupling between trophic groups via predation, coprophagy, and other mechanisms. In reefs where fish assemblages are altered by the removal of important functional groups such as top predators and other large-bodied species, linkages between feces and food are probably less tight (Sandin *et al.*, 2008). Feces may remain suspended in water as particles or become entrained into benthic substrates, and biogeochemical processes in 'water' and 'sediments' may be substantially influenced by the metabolic activities of highly abundant feces-associated bacteria. Coupled investigations of fish assemblage trophic structures and reef-based microbial dynamics may reveal unrecognized functional linkages. Fish feces represent an integration of ingested prey, and the deposition of fish feces may be a mechanism by which bacteria are transported within coral reef ecosystems.

Acknowledgements

We thank J. Nguyen for assistance with DGGE analyses and culturing, E.A. Allen and P. Narasingarao for advice on phylogenetic analyses, and B. Deck for assistance with composition analyses. Support was provided to F.A. by the GEF Coral Reef Targeted Research Program, a National Science Foundation grant (OCE0648116), and The Gordon

and Betty Moore Foundation. Further support was provided by The Nature Conservancy at Palmyra Atoll, and an NSF Graduate Student Fellowship to S.S. Sample collection was permitted via the US Fish and Wildlife Service. We thank O. Pantos, J. Smith, J. Osbourne, C. McDonald, B. Ruttenberg, S. Walsh, M. Vermeij, S. Hamilton, and E. Sala for assistance with sample collection and processing.

References

Angert ER, Clements KD & Pace NR (1993) The largest bacterium. *Nature* **362**: 239–241.

Bailey TG & Robertson DR (1982) Organic and caloric levels of fish feces relative to its consumption by coprophagous reef fishes. *Mar Biol* **69**: 45–50.

Bidle KD & Azam F (2001) Bacterial control of silicon regeneration from diatom detritus: significance of bacterial ectohydrolases and species identity. *Limnol Oceanogr* **46**: 1606–1623.

Clements KD (1997) Fermentation and gastrointestinal microorganisms in fishes. *Gastrointestinal Microbiology, Vol. 1* (Mackie RI, White BA & Isaacson RE, eds), pp. 156–198. Springer, Berlin.

Clements KD & Raubenheimer D (2006) Feeding and nutrition. *The Physiology of Fishes* (Evans DH & Claiborne JB, eds), pp. 47–82. Taylor and Francis Group, Boca Raton, FL.

Clements KD, Pasch IBY, Moran D & Turner SJ (2007) Clostridia dominate 16S rRNA gene libraries prepared from the hindgut of temperate marine herbivorous fishes. *Mar Biol* **150**: 1431–1440.

Crosby LD & Criddle CS (2003) Understanding bias in microbial community analysis techniques due to rrn operon copy number heterogeneity. *Biotechniques* **34**: 790–802.

Dunlap PV & Steinman HM (1986) Strain variation in bacteriocuprein superoxide dismutase from symbiotic *Photobacterium leiognathi*. *J Bacteriol* **165**: 393–398.

Fang H, Chen C-Z & Zhang X-J (2005) Studies on biological properties of *Vibrio qinhuangdaora* sp. nov. isolated from diseased flounder *Paralichthys olivaceus*. *Acta Hydrob Sin* **29**: 366–371.

Felsenstein J (2005) *PHYLIP (Phylogeny Inference Package) version 3.6*. Distributed by the author, Department of Genome Sciences, University of Washington, Seattle.

Ferrari VC & Hollibaugh JT (1999) Distribution of microbial assemblages in the Central Arctic Ocean Basin studied by PCR/DGGE: analysis of a large data set. *Hydrobiologia* **401**: 55–68.

Fishelson L, Montgomery WL & Myrberg AA (1985) A unique symbiosis in the gut of tropical herbivorous surgeonfish (Acanthuridae, Teleostei) from the Red Sea. *Science* **229**: 49–51.

Garren M, Smriga S & Azam F (2008) Gradients of coastal fish farm effluents and their effect on coral reef microbes. *Environ Microbiol* **10**: 2299–2312.

Hoppe HG (1983) Significance of exoenzymatic activities in the ecology of Brackish water – measurements by means of methylumbelliferyl substrates. *Mar Ecol-Prog Ser* **11**: 299–308.

Jacobsen TR & Azam F (1984) Role of bacteria in copepod fecal pellet decomposition – colonization, growth rates and mineralization. *B Mar Sci* **35**: 495–502.

Jensen S, Frost P & Torsvik VL (2009) The nonrandom microheterogeneity of 16S rRNA genes in *Vibrio splendidus* may reflect adaptation to versatile lifestyles. *FEMS Microbiol Lett* **294**: 207–215.

Lawrence SG, Ahmad A & Azam F (1993) Fate of particle-bound bacteria ingested by *Calanus pacificus*. *Mar Ecol-Prog Ser* **97**: 299–307.

Ley RE, Hamady M, Lozupone C *et al.* (2008) Evolution of mammals and their gut microbes. *Science* **320**: 1647–1651.

Lunau M, Lemke A, Walther K, Martens-Habbena W & Simon M (2005) An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. *Environ Microbiol* **7**: 961–968.

Macian MC, Garay E, Grimont PAD & Pujalte MJ (2004) *Vibrio ponticus* sp. nov., a neighbour of *V. fluvialis–V. furnissii* clade, isolated from Gilthead sea bream, mussels and seawater. *Syst Appl Microbiol* **27**: 535–540.

Meyer JL & Schultz ET (1985) Migrating haemulid fishes as a source of nutrients and organic matter on coral reefs. *Limnol Oceanogr* **30**: 146–156.

Muyzer G, Dewaal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S ribosomal RNA. *Appl Environ Microb* **59**: 695–700.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig WG, Peplies J & Glockner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**: 7188–7196.

Rimmer DW & Wiebe WJ (1987) Fermentative microbial digestion in herbivorous fishes. *J Fish Biol* **31**: 229–236.

Robertson DR (1982) Fish feces as fish food on a Pacific coral reef. *Mar Ecol-Prog Ser* **7**: 253–265.

Sandin SA, Smith JE, DeMartini EE *et al.* (2008) Baselines and degradation of coral reefs in the northern line islands. *PLoS ONE* **3**: e1548.

Schloss PD & Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microb* **71**: 1501–1506.

Smith DC, Simon M, Alldredge AL & Azam F (1992) Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. *Nature* **359**: 139–142.

Stevens CE & Hume ID (1998) Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. *Physiol Rev* **78**: 393–427.

Sutton DC & Clements KD (1988) *Aerobic, Heterotrophic Gastrointestinal Microflora of Tropical Marine Fishes, Proceedings of the Sixth International Coral Reef Symposium, Vol. 3* (Choat JH *et al.*, eds), pp. 185–190. Sixth International Coral Reef Symposium Executive Committee, Townsville, Qld, Australia.

Thompson FL, Iida T & Swings J (2004) Biodiversity of vibrios. *Microbiol Mol Biol Rev* **68**: 403–431.

Thompson JR, Marcelino LA & Polz MF (2002) Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. *Nucleic Acids Res* **30**: 2083–2088.

Wang Q, Garrity GM, Tiedje JM & Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**: 5261–5267.

Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**: 697–703.

Wotton RS & Malmquist B (2001) Feces in aquatic ecosystems. *Bioscience* **51**: 537–544.

Xie ZY, Hu CQ, Zhang LP, Chen C, Ren CH & Shen Q (2007) Identification and pathogenicity of *Vibrio ponticus* affecting cultured Japanese sea bass, *Lateolabrax japonicus* (Cuvier in Cuvier and Valenciennes). *Lett Appl Microbiol* **45**: 62–67.

Chapter 2, in its entirety, is a reprint of the material as it appears in: Smriga S, Sandin S, and Azam F (2010) Abundance, diversity, and activity of microbial assemblages associated with coral reef fish guts and feces. *FEMS Microbiology Ecology* **73**: 31-42. The dissertation author was the primary investigator and principal author of this manuscript.

CHAPTER 4: Persistence of parrotfish feces derived bacteria in coral reefs

ABSTRACT

Fish feces deposition in coral reef ecosystems enables movement of particulate organic matter and may provide a transport mechanism for associated microbes. Previous phylogenetic analyses of feces-derived 16S rRNA gene clone libraries revealed a distinct fish feces-associated vibrio-like taxonomic group (FFV-L) that is related to Vibrionaceae but is distinct from *Vibrio* spp. and *Photobacterium* spp. In this chapter, I show that FFV-L have low similarity (≤92%) to 16S rRNA genes in Global Ocean Survey metagenomic databases, which reinforces the 'uniqueness' of the taxon. PCR primers specific to FFV-L were designed and used to screen 'aged' parrotfish feces samples collected over several days during 2006 and 2008 at Palmyra Atoll. Many feces were FFV-L positive, which suggests that excreted parrotfish feces enable persistence or survival of FFV-L in the environment. FFV-L were also detected in mucus from *Montipora* sp. and *Acropora* sp. corals that were dosed with parrotfish feces during short-term *in situ* challenge experiments, demonstrating the potential for FFV-L to interact with coral mucus-associated bacteria. Meanwhile, community composition analyses via denaturing gradient gel electrophoresis (DGGE) revealed that at least two bands (phylotypes) commonly occurred among different samples collected each year, which suggests that some non-FFVL phylotypes also occur consistently in aged feces. I suggest that FFV-L and other distinct bacterial phylotypes have adapted to use parrotfish feces as a habitat for survival and/or to utilize fecal organic matter for growth.

INTRODUCTION

Microbes (Bacteria and Archaea) drive biogeochemical processes in temperate coastal ecosystems (Azam et al., 1994) including tropical coral reef waters (e.g. Wild et al., 2004). Filter feeding by benthic organisms and fish herbivory are among the other processes that greatly influence carbon and nutrient fluxes in coral reefs. For example, some corals, sponges, and bivalves can filter reef water and incorporate particulate and dissolved organic matter presumably as a source of nutrition (Yahel et al., 2003; Houlbreque et al., 2004; Yahel et al., 2009). Similarly, fish feces can be a source of nitrogen and phosphorous for mobile benthic invertebrates (Pinnegar and Polunin, 2006) and corals (Meyer and Schultz, 1985). These 'macroscale' interactions between benthic organisms (e.g. corals) and biologically-derived components in the water (e.g. fish feces) may host a nuanced set of 'microscale' interactions occurring between specific bacterial taxa and the distinct physical components with which they consistently associate.

In a previous study I found that feces and gut contents from three coral reef fish species contained highly abundant microbial assemblages (see Chapter III). Bacteria associated with parrotfish feces were capable of rapid growth and may have contributed to hydrolytic enzyme activity associated with feces. Microbial communities in parrotfish feces were comprised mostly of *Vibrio* spp. and *Photobacterium* spp., but they also hosted phylotypes from a distinct taxonomic group. The group, henceforth referred to as the fish feces-associated vibrio-like taxonomic group (FFV-L), contains representatives from feces of parrotfish,

surgeonfish, and cultured milkfish from the Philippines (Garren et al., 2008). The putative phylogenetic alignment for FFV-L is within the order Vibrionaceae and is more similar to the genera *Enterovibrio* and *Salinivibrio* than to *Vibrio* or *Photobacterium* (Smriga et al., 2010). However, its least derived representative sequence is only 95% similar to *Enterovibrio coralii*. Thus FFV-L bacteria may be relatively unique.

It is useful to explore the distribution of distinct bacterial groups like FFV-L since it may trace the fate and distribution of feces-associated bacterial assemblages. FFV-L may transfer into benthic organisms such as corals and may be capable of long-term persistence in the environment. The ability of cells to persist could be enhanced by attachment to mobile organic particles and subsequent transport in water. I have observed a phenomenon that may be relevant to bacterial persistence, which is the formation of 'feces-mucus-bundles.' In response to the settlement of feces onto their surfaces, some corals undergo copious mucus production and the mucus entraps fecal particulates (see Chapter V). Over time the bundles become buoyant, separate from the corals, and are able to move with currents in the water column. The bundles have potential to incorporate bacteria from feces, coral mucus, and/or seawater, and the metabolic activities of the bacteria could influence bundle degradation.

This chapter addresses the hypotheses that FFV-L and other bacteria associated with parrotfish feces: a) occur consistently over days to years and can persist in the environment; and b) can become entrained into 'feces-mucus-bundles.' It describes the presence of FFV-L inferred from use of primers specific to this group and

describes the co-occurrence of other phylotypes inferred from DGGE analyses. It also addresses bacteria abundances associated with aged feces. The study provides a foundation for formulating further hypotheses about the functional role(s) of microbes within macroecological interactions among fish, corals, and particulate organic matter.

MATERIALS AND METHODS

Sample collection

Samples were collected from coral reef sites at Palmyra Atoll (5°53′N 162°05′W) during September and October 2008. Feces from the parrotfish *Chlorurus sordidus* were collected at the Western Terrace, which are a series of backreef sites that comprise the western end of the atoll. Fish were not harvested due to regulatory permitting constraints. Various sample types were collected (Table 1) and care was taken to minimize the collection of benthic substrate that might contaminate feces samples. Feces from *C. sordidus* were granular and composed primarily of coral-derived carbonates and inorganic sediments. Small fragments of red- and green-algae were observed within the granular carbonate matrix. All sample aliquots were preserved in tubes containing RNAlater™ (Ambion) for DNA analyses or 0.2-μm filter-sterilized 2% formaldehyde-seawater for microscopy. Preserved samples were stored immediately at -20°C.

Cell abundances in feces

Bacteria abundances were determined for feces using epifluorescence microscopy as previously described (Smriga et al., 2010). An aliquot of ‘aged’ feces

collected each day was incubated in seawater by distributing ~50 mg dry weight portion into each of three 1 L aquaria. Feces collected on successive days were added without removal of the prior days' feces, so accumulation was additive. On the fourth day, 12 h after addition of that day's feces, the accumulated particles were removed from each aquarium, fixed in 2% formaldehyde, and stored at -20°C until processing to determine cell abundance.

Design of FFV-L specific primers

The online NCBI tool Primer-BLAST was used to identify putative FFV-L specific primers. One sequence from *C. sordidus* feces (Acc No. AJ123456; Smriga et al., 2010) was used as the target template. Two primers (224F and 1298R) were identified with the following sequences: 224F (5'-TTC GGA CCT TCC GCA CTG GG-3') and 1298R (5'-CGC TTC ACC TCG CGG TCT CG-3'). The numbering corresponds to the *E. coli* 16S rRNA gene. In screening environmental DNA extracts, presence (+) of FFV-L indicates a PCR product of ~1072 nt was observed on an agarose gel.

PCR amplification of 16S rRNA genes

DNA was extracted from feces, coral mucus, and feces-mucus-bundle samples using the UltraClean^{TM} Soil Kit (MoBio). PCR amplification of 16S rRNA genes for DGGE analyses followed a nested approach as described previously (Smriga et al., 2010) using GC-clamped 341F and 534R primers. PCR amplification for clone

libraries using FFV-L specific primers were 30-cycles of amplification (1 min at 94°C; 1 min at 52°C; 3 min at 72°C) followed by a 10 min extension at 72°C. FFV-L products were observed on 1.5% agarose gels. PCR amplification of 16S rRNA genes for clone libraries used primers 27F and 1492R was as described previously (Smriga et al., 2010). The 27F/1492R amplicons were from total extracted DNA of 'BrdU mucus' and 'BrdU bundles' samples, i.e. amplification was not performed on immunocaptured fractions of BrdU-labeled DNA (Table 1).

Cloning and sequencing

PCR products (~200 nt length for excised DGGE bands; ~1072 nt for FFV-L amplicons) were cloned as previously using the Invitrogen pCR4-TOPO Kit (Smriga et al., 2010). Plasmid-specific primers M13F/M13R were used to amplify inserts, and the products were purified (MolBio UltraClean PCR Clean-up Kit) and submitted to a commercial service for sequencing using primers T3/T7 (GeneWiz Inc., La Jolla, CA). Among clone libraries containing FFV-L amplicons, the portion of clones that contained putative FFV-L sequences ranged 25 to 100%.

Denaturing gradient gel electrophoresis (DGGE) analyses

Aliquots of PCR products (amplified via 341F/534R) were separated by GC-content via DGGE as previously described (Smriga et al., 2010). A selection of DGGE bands were excised from gels and eluted into saline-sodium citrate (SSC) buffer. A subset of the eluted bands were reamplified via PCR and ran on a new DGGE gel to

check the migration location. Select bands were cloned using the pCR4-TOPO kit (Invitrogen) and four to eight clones from each library were selected for sequencing.

Sequence analyses

Sequence trace files were trimmed manually using Sequencher 4.5 (Gene Codes Corp). All 16S rRNA gene sequences were queried against the NCBI nt database to determine putative identification. All sequences were checked for chimera potential using Bellopheron 3 (Huber et al. 2004). A representative FFV-L sequence was compared by BLAST to several metagenomic databases using the Community Cyberinfrastructure for Advanced Marine Microbial Ecology Research and Analysis (CAMERA). Some databases contain Sanger-derived sequences which are generally >700 nt in length while other databases contain pyrosequencing- ('454') derived sequences which are generally <500 nt in length. High similarity matches to longer sequences will have better e-values than high similarity matches to shorter sequences and therefore can be interpreted as a more accurate representation of true taxonomic identity. The top three similarity matches for each database were reported.

Transplantation of feces onto Montipora and Acropora corals

Parrotfish feces were transplanted onto live encrusting *Montipora* and plating *Acropora* colonies to test the short-term response of bacterial communities associated with coral mucus. A one hour experiment on *Montipora* was performed in the morning (October 9, 2008) and a separate one hour experiment was performed on

Acropora in the afternoon. The transplanted feces used to dose corals were collected from accumulated piles on the reef bottom (i.e. 'aged' feces). To start the experiment, feces were dosed onto three locations of a single coral colony resulting in piles ~2 cm in diameter and ~1 cm in height. The following samples were collected 5, 30, and 60 min after dosing: coral mucus immediately adjacent to the dosing location ('target mucus'), coral mucus 10 cm away from the dosing location ('10 cm mucus'), and the aggregation of feces and coral mucus produced directly at the dosing site ('feces-mucus-bundles'). A different dosing site was used for each timepoint to minimize disturbance to any single location on the coral colony.

RESULTS

Bacteria abundances in 'aged' feces

Mean bacteria abundance was 5.55×10^9 ($\pm 0.43 \times 10^9$) (g dry weight)$^{-1}$ among 'aged' feces samples collected from the field over four days in 2008. After 4 d further incubation of aged feces in seawater aquaria (see Methods), mean bacteria abundance was 6.24×10^9 ($\pm 0.48 \times 10^9$) (g dry weight)$^{-1}$ (n=3).

Uniqueness of FFV-L 16S rRNA gene

In BLAST comparisons of a representative FFV-L sequence to nucleotide metagenomic databases, the nearest similarity match was 92% among Global Ocean Survey 16S rRNA sequences as well as all metagenomic non-coding RNAs (Table 2). The best match to a human distal gut database was 89%, to the 2005 Line Islands database was 94%, and to the 'all metagenomic' database was 97% (Table 2).

Generally, BLAST scores were >1000 for similarities to Sanger-based sequences and <800 for 454-based sequences.

Presence of FFV-L in feces samples from 2006 and 2008

Using the FFV-L 16S rRNA gene specific primers (224F/1298R), PCR amplicons were attained from several aged feces samples collected in 2008 covering several days (Table 3). Among clone libraries constructed from feces FFV-L amplicons, the percentage of clones that appeared on agarose gels to have the correct insert length (~1072 nt) ranged 25% to 100%. The remaining clones contained smaller inserts that ranged ~300 to ~900 nt. For the clones that appeared to contain the correct insert length, sequence data showed that most were exactly 1072 nt. All such sequences were >98% similar to FFV-L sequences via BLAST comparison to the GenBank nt database, i.e. they were FFV-L sequences. In contrast, some clone sequences were actually slightly longer or shorter than 1072 nt, and these always contained the 1298R primer at both ends and had no similarity hits to GenBank. In other words, they were 'nonsense' sequences that were 'double-primed' with 1298R. Meanwhile, no amplicons were attained either from seawater or from 2006 feces (Table 3), with the latter result most likely due to degradation of preserved DNA.

Presence of FFV-L after transplanting feces onto corals

FFV-L were detected in *Acropora* feces-mucus-bundles and 'target' mucus (immediately adjacent to the feces site) at 5, 30, and 60 min following dosing with

transplanted parrotfish feces. Similarly, FFV-L sequences were detected in *Montipora* feces-mucus-bundles at 5 min and 60 min post-dosing and in target mucus at 30 min post-dosing, though PCR amplification was weak for the latter (Table 4). Conversely, FFV-L was detected in mucus collected 10 cm away from the dosing site up to 60 min in *Montipora* mucus and up to 30 min in *Acropora* mucus (Table 4), though it was detected at 60 min in *Acropora* mucus.

Co-occurrences of phylotypes in DGGE profiles of feces from 2006 and 2008

Many bands in DGGE profiles of feces samples (amplified using domain-specific primers 341F/534R) were specific to each sample (Fig. 1). However, at least two bands occurred commonly across DGGE profiles of feces from 2006 and 2008. One band occurred at ~46% denaturant (i) and a second band occurred at ~53% denaturant (ii) in a gel ranging 30% to 60% denaturant (Fig. 1). The bands were observed in sample profiles on multiple different gels (not shown). Samples in which these two common bands were observed include: 2006 feces (-3 and -5), 2008 feces (Oct4, Oct5, Oct9-3, and Oct10-3), FE2 feces, and FE3 feces.

The taxonomic identities of bands (i) and (ii) excised from feces profiles were generally Vibrionaceae and Alteromonadales (Table 5). Definitive identities for any single band could not be determined because multiple clones derived from any single band were not identical. For example, two phyla and at least four genera were represented among eight clones in a library derived from band (ii) of the 2008 feces sample Oct4 (Table 5), and the %GC was not the same among the clones, i.e. it ranged

53.1% to 55.7% for this band (Table 5). Nonetheless, some taxa occurred commonly in multiple samples for both bands. For example, *Vibrio* spp. were observed in band (i) of the 2006 sample feces-3 and band (i) of the 2008 sample FE2 (Table 5). Also, *Pseudoalteromonas* spp. were observed in band (ii) of the 2006 sample feces-3 and band (ii) of the 2008 sample Oct4 (Table 5). Notably, all clones for some bands were highly similar (e.g. four clones for sample 2008 FE2 were *Vibrio* sp.) while clones for other bands were so divergent that they represented different phyla (e.g. four clones for 2006-3 represented gamma- and beta-proteobacteria as well as Cytophaga).

Comparison of microbial assemblages in coral mucus and feces-mucus-bundles

16S rRNA gene clone libraries for coral mucus and feces-mucus-bundles (amplified using domain specific primers 27F/1492R) were generally the same at the phylum level in that Proteobacteria dominated, comprising 36 of 41 clones (88%) in the bundle library and 31 of 43 clones (72%) in the mucus library. Significant differences existed only at the genus level within the gamma-Proteobacteria. The bundle library had more *Thalassomonas* spp. sequences (9 of 41 while there were zero in mucus, $p = 0.00153$), and the mucus library had more *Alteromonas* spp. sequences (11 out of 43 while there was one out of 41 in bundles, $p = 0.0044$).

DISCUSSION

The distinct bacterial phylotype FFV-L was consistently associated with parrotfish feces at Palmyra Atoll (Table 3). FFV-L bacteria represent a taxonomic

group that apparently has not previously been observed. Sequences obtained with FFV-L specific primers matched with low similarity to any in the GOS database (Table 2), and given the vast genetic content represented in GOS, the database acts as a 'gold standard' for marine microbial 16S rRNA gene diversity. FFV-L sequences found in the environment may therefore be an indicator for the presence of residual fish feces. Indeed, the results from short-term field experiments showed FFV-L *was not* detected in coral mucus 10 cm away from a feces dosing spot (except in *Acropora* coral after 60 min) while it *was* detected in mucus immediately adjacent to the dose site and in mucus produced in response to dosing. Furthermore, FFV-L was not detected via PCR in seawater at Palmyra (Table 2) or in coastal California seawater (result not shown). The lack of FFV-L in Palmyra seawater is consistent with rapid sinking and settlement of parrotfish feces, though it cannot be ruled out that FFV-L were present in seawater at abundances so low that they were not detectable via PCR. FFV-L has not yet been identified among cultured bacterial isolates from parrotfish feces, though it remains an important goal.

Feces samples collected over five days in 2006 and 13 days in 2008 shared at least some common phylotypes as indicated by two commonly occurring bands in DGGE profiles (Fig. 1). The phylotypes were not restricted to a single location at Palmyra since the 2006 collection site (Penguin's Pit) was ~2 km distant from the 2008 collection site (Western Reef Terrace). Similar patterns, i.e. recurrences of bacterial communities over time scales of years and in sampling locations separated by m to km, have been observed in other coastal marine ecosystems. For example,

assemblage compositions in coastal California seawater experienced seasonal and annual reoccurrences (Fuhrman et al., 2006). Also, assemblage compositions were similar in benthic sediments that were separated by kilometers (Hewson et al. 2007). In these studies, abiotic and biotic environmental factors influenced the structure of overall assemblage composition while specific phylotypes (OTUs) occurred consistently at low levels. This is congruous with my observation that feces DGGE profiles contained many bands that were unique to each sample with a few that occurred commonly.

The presence of FFV-L (Table 3) and common DGGE bands in 'aged' feces collected over several days (Fig. 1) suggests these phylotypes may be able to persist in fecal aggregates post-egestion in the environment. In this regard, abundance data provide insight as to whether bacteria have potential to utilize feces for growth. Bacteria abundance in 'aged' feces collected from the field (5.55×10^9 (g dry wt feces)$^{-1}$) increased only slightly upon 4 d further incubation in aquaria (6.24×10^9 g^{-1})(see Results). Thus, the bacterial carrying capacity for parrotfish feces may be ~6×10^9 g^{-1}. In comparison, 'fresh' feces collected in 2006 that were preserved ~3 h post-egestion contained 1.71×10^9 cells g^{-1} while aliquots of the same feces that were experimentally incubated another 10 h contained 3.59×10^9 g^{-1} (see Chapter III).

During the 2006 experiments, the apparent carrying capacity (~6×10^9 g^{-1}) was not reached within 12 h post-egestion, but given growth rates of 1.88×10^8 new bacteria (g dry wt)$^{-1}$ h^{-1} (see Chapter III), the carrying capacity may have been reached ~23 h post-egestion. My ability to further constrain this time estimate for population

abundances 'leveling off' could be strengthened if I repeated incubations with 'fresh' feces for longer or if I knew the amount of time that 'aged' feces had remained settled in the reef before it was collected.

I do not know which phylotypes experienced growth nor whether cells utilized particulate or dissolved fractions of the fecal particles as growth substrate. Future studies should utilize fluorescence in situ hybridization (FISH) microscopy to quantify the abundance and dynamics of FFV-L and other specific taxa. Perhaps only certain phylotypes grew while most used feces passively as habitat for survival. Regardless, an abundance of ~6 x 10^9 g^{-1} is one to two orders of magnitude lower than those observed in feces of snapper and surgeonfish (see Chapter II), which suggests that parrotfish feces may be nutrient limited relative to feces of other fishes. It may be that parrotfish feces act as reservoirs for inactive bacteria until pulses of nutrients from exogenous sources enable growth.

So far we have limited constraints on the physicochemical conditions that are ideal for long-term proliferation or survival of feces-associated bacteria. However, certain fecal bacteria may be adapted for survival within a range of diverse conditions including associations with microalgae, coral mucus, seawater (as free-living planktonic organisms), or other reef components. Such adaptations may be critical for those bacteria that originate in the environment, become ingested and survive passage through the gastrointestinal tract, and then are egested with feces. These cells would need to be adapted to survive low pH in the stomach, which ranges 1.9 to 4.3 in herbivorous fishes, over variable gut residence times, which range 4 to 6 h for marine

herbivorous fishes by some estimates and 12 h or greater by others (Clements and Raubenheimer, 2006). Microbes would then experience an increase in pH as they were egested into seawater (pH~8.1). Regardless of a microbes's capacity to survive such diverse conditions, growth may occur only in specific microenvironments.

At the spatial scales relevant to bacteria, parrotfish fecal aggregates comprise a distinct microhabitat in the reef, an ecosystem-within-an-ecosystem. Future efforts that address transport mechanisms of parrotfish feces and feces-mucus-bundles in reefs may reveal functional role(s) for feces-associated bacteria in coral reef health. The data presented here and previously (see Chapters II and III) suggest most fish feces bacteria are gamma-proteobacteria that fall into the orders Alteromonadales and Vibrionales. These and other bacterial taxa that consistently associate with fish feces have potential to drive biogeochemical processes such as carbon turnover. They may also influence aggregate dissociation and aid disbursement of fecal particles into the water column. Moreover, algae and other fragments containing high caloric content that are released from dissociated feces could become available as food for macrofauna and incorporated into reef food webs.

Table 4-1: Description of samples collected from Palmyra Atoll.

Sample Names	Description	Approximate # replicates
2006 feces	Feces from individual *C. sordidus* collected immediately post-egestion using sterile syringes. One individual feces was collected each day over five days. Samples remained at ambient temperature (~26°C) for ~10 h before preservation (Smriga et al., 2010).	5
2008 feces	'Aged' feces collected from accumulated piles on the reef bottom, primarily at fish 'dumping' stations. Nearly entirely composed of *C. sordidus* feces but may contain contributions from other fish species that defecate granular sediments. Samples were collected each day over ~13 days during 2008 and used to dose coral fragments in tank experiments. Thus, they aged further from the time they were collected (~10 am daily) until they were preserved with 2% formaldehyde (~11 pm daily).	13
BrdU feces BrdU bundles BrdU mucus BrdU SW	The following were collected in replicate for incubation with bromodeoxyuridine: aged parrotfish feces accumulated on the reef bottom (BrdU feces); aged naturally-occurring feces-mucus-bundles from two separate *Acropora* plating colonies (BrdU bundles); coral mucus from the same two colonies (BrdU mucus); and seawater ~2 m above each coral (BrdU SW). Samples were collected in replicate at noon on October 13, 2008, at the Western Terrace using sterile 60 mL syringes. They were brought to the surface, diluted in surface seawater to 500 mL in a sterile polycarbonate Nalgene bottle, amended with BrdU to 20 nM, and incubated 5 h in the dark at ambient temperature (~28°C). Incubations were stopped by placing the bottles on ice where they remained for ~7 h. The replicate aliquots were then filtered sequentially: first 300 mL were collected onto 10 μm pore-size (25 mm diameter) polycarbonate membranes, and then 50 mL of the resulting filtrate onto 0.2 μm pore-size membranes. The membranes were stored in individual 1 mL RNAlater™ (Ambion) aliquots at -20°C until processing (~15 months). These samples were prepared to enable the eventual identification of actively growing phylotypes via immunocapture of BrdU-labeled bacterial DNA. In this study, the total extracted DNA (not the BrdU-labeled fractions) was used for analyses.	2 per sample type
FE2 feces *Acropora*: target-mucus 10-cm-mucus bundles	The following were preserved during Field Experiment 2: 'aged' feces collected from accumulated piles on the reef bottom used to dose the surface of a plating *Acropora* colony (FE2 feces); coral mucus collected immediately adjacent to the dosing spot (*Acropora* target-mucus); coral mucus collected 10 cm away from the feces dosing spot (*Acropora* 10-cm-mucus); and feces-mucus-bundles that formed in response to dosing (*Acropora* bundles).	1 for feces; 3 per other sample types
FE3 feces *Montipora*: target-mucus 10-cm-mucus bundles	Samples for Field Experiment 3 were the same as above except the dosing was on an encrusting *Montipora* coral colony.	1 for feces; 3 per other sample types

Table 4-2: BLAST comparison of a representative FFV-L 16S rRNA gene sequence to metagenomic databases via the Community Cyberinfrastructure for Advanced Marine Microbial Ecology Research and Analysis (CAMERA).

CAMERA database	Database sequence ID	Identities (similarity)	BLAST score	Sample Type	Sample Location
GOS Site-specific 16S sequences	JCVI_16S_73182	704/764 (92%)	1039	Seawater, open ocean	Sargasso 11
	JCVI_16S_187270	704/764 (92%)	1039	Seawater, open ocean	Sargasso 11
	JCVI_16S_279138	704/764 (92%)	1039	Seawater, open ocean	Sargasso 11
All Metagenomic ncRNAs	JCVI_NT_1113981742445	884/960 (92%)	1277	Whalefall rib bone	Santa Cruz Basin
	JCVI_NT_1112674871595	711/767 (92%)	1045	Seawater, coral reef	Moorea, Cooks Bay
		704/764 (92%)	1039	Seawater, open ocean	Sargasso 11
HumanDistalGut: All Metagenomic Shotgun Reads	READ_00078333	439/492 (89%)	548	Human stool	Human distal gut
	READ_00005943	299/345 (86%)	304	Human stool	Human distal gut
	READ_00005943	175/192 (91%)	246	Human stool	Human distal gut
LineIsland: All Metagenomic 454 Reads	READ_01150077	94/100 (94%)	151	Seawater, coral reef	Palmyra
	READ_02376515	97/107 (90%)	133	Seawater, coral reef	Kiritimati
	READ_02440401	74/79 (93%)	118	Seawater, coral reef	Kiritimati
All Metagenomic 454 Reads	READ_00410770	461/475 (97%)	823	Seawater	Western Engl Channel
	READ_00402657	473/501 (94%)	772	Seawater	Western Engl Channel
	READ_00401954	468/501 (93%)	692	Seawater	Western Engl Channel

Table 4-3: PCR amplification using FFV-L specific primers. An amplicon was considered FFV-L –positive if it matched with >98% similarity to FFV-L sequences when compared by BLAST to the GenBank nt database.

Sample ID	Sample type	# of samples screened	# of samples FFV-L PCR positive	Confirmed to contain at least one FFV-L clone
2008-feces	Aged feces	8	4	3
2006-feces	Fresh feces	5	0	n/a
BrdU-Bun	Bundle	2	1	1
BrdU-feces	Aged Feces	2	1 (smeered band)	n/a
BrdU-SW-0.2	Seawater	2	0	n/a
BrdU-SW-10	Seawater	2	0	n/a

Table 4-4: Presence or absence of FFV-L sequences in coral mucus after transplanting parrotfish feces onto coral surfaces. FFV-L specific PCR primers (224F/1298R) were used to screen samples. FFV-L were also detected in the feces aliquots used to dose coral surfaces.

	Sample Type	PCR amplification (-/+)		
		5 min.	30 min.	60 min.
Montipora	Target mucus	-	+/-	-
	10 cm mucus	-	-	-
	Feces-Mucus-Bundles	++	N/A	+
Acropora	Target mucus	+	+	+++
	10 cm mucus	-	-	+
	Feces-Mucus-Bundles	+	++	+++

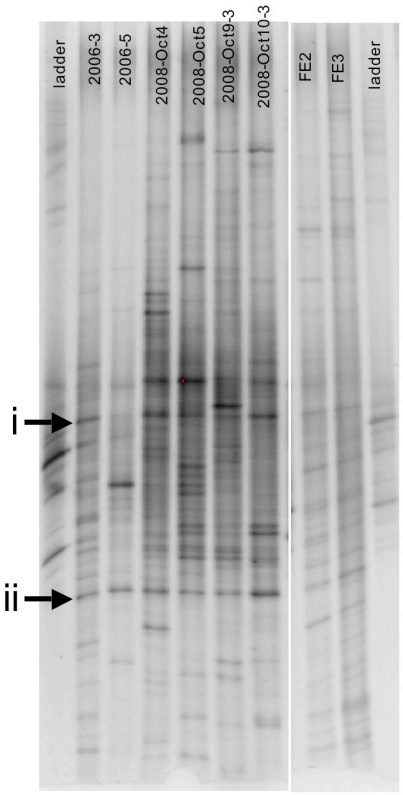


Figure 4-1: Representative DGGE profiles for multiple parrotfish feces samples in a 30 – 60% denaturant gel. Two bands occurred commonly, one at ~46% denaturant (i) and the second at ~53% denaturant (ii). Other bands were either specific to each sample or occurred in two to four other samples. PCR products (~200 nt) were produced using primers specific to domain Bacteria (341F/534R).

Table 4-5: Identities of individual DGGE bands (i) and (ii) determined from multiple clones per band. The bands were extracted from DGGE profiles of several parrotfish feces samples. The percentages are approximate location on a gel that ranged 30 - 60% denaturant.

Feces Sample ID	DGGE band	clone ID	%GC	Summary of top 25 matches to GenBank via BLAST	
				Phylum-level	Genus-level
2006-3	i (46%)	1-3	52.6	gamma-Proteobacteria	*Alteromonas* sp.
		1-4	55.7	beta-Proteobacteria	Uncultured beta-proteobacterium
		1-7	54.1	gamma-Proteobacteria	*Vibrio* sp. (non FFV-L)
		1-8	52.9	Cytophaga	*Persicobacter* sp.
2008-OCT10-E	i (46%)	6-1	52.7	Cytophaga	*Persicobacter* sp.
		6-3	49.0	Firmicutes	Uncultured Firmicutes
		6-4	53.4	gamma-Proteobacteria	*Shewanella/Enterovibrio*/FFV-L spp.
		6-6	52.7	Cytophaga	*Persicobacter* sp.
		6-8	53.6	gamma-Proteobacteria	*Vibrio/Enterovibrio*/FFV-L spp.
2008 FE2	i (46%)	7-2	53.1	gamma-Proteobacteria	*Vibrio* spp. (non FFV-L)
		7-3	53.1	gamma-Proteobacteria	*Vibrio* spp. (non FFV-L)
		7-6	55.2	gamma-Proteobacteria	*Vibrio* spp. (non FFV-L)
		7-7	53.6	gamma-Proteobacteria	*Vibrio* spp. (non FFV-L)
2006 feces-3	ii (53%)	11-4	55.4	gamma-Proteobacteria	*Pseudoalteromonas* sp.
		11-5	53.6	gamma-Proteobacteria	FFV-L (uncultured)
		11-13	50.3	gamma-Proteobacteria	FFV-L (uncultured)
2008 FE2	ii (53%)	17-1	54.6	gamma-Proteobacteria	*Vibrio* spp. (non FFV-L)
		17-8	53.1	gamma-Proteobacteria	*Shewanella/Colwellia* spp.
		17-9	53.1	gamma-Proteobacteria	FFV-L (uncultured)
		17-10	54.3	gamma-Proteobacteria	FFV-L (uncultured)
		17-11	53.1	gamma-Proteobacteria	*Vibrio* spp.
		17-12	53.1	gamma-Proteobacteria	*Shewanella/Colwellia* spp.
2008-Oct4	ii (53%)	13-6	55.1	delta-Proteobacteria	*Bacteriovorax* spp.
		13-7	53.8	gamma-Proteobacteria	*Vibrio* spp. (non FFV-L)
		13-8	55.1	delta-Proteobacteria	*Bacteriovorax* spp.
		13-9	53.1	gamma-Proteobacteria	*Colwellia/Shewanella/Ferrimonas* spp.
		13-10	53.1	gamma-Proteobacteria	*Colwellia/Shewanella/Ferrimonas* spp.
		13-11	53.7	gamma-Proteobacteria	*Pseudoalteromonas* spp.
		13-12	53.1	gamma-Proteobacteria	*Colwellia/Shewanella/Ferrimonas* spp.
		13-13	55.7	delta-Proteobacteria	*Bacteriovorax* spp.

Acknowledgements

I thank P. Narasingarao and E. Eloe for advice on primer design and phylogenetic analyses, respectively. Support was provided to FA by the GEF Coral Reef Targeted Research Program, a National Science Foundation grant (OCE0648116), and The Gordon and Betty Moore Foundation. Further support was provided by The Nature Conservancy at Palmyra Atoll, and an NSF Graduate Student

Fellowship to S. Smriga. Sample collection was permitted via the U.S. Fish and Wildlife Service. I also thank J. Smith, S. Hamilton, S. Sandin, N. Price, M. Garren, J. Baum, T. Davies for sample collection and assistance at Palmyra Atoll.

REFERENCES

Azam, F., Smith, D.C., Steward, G.F., and Hagstrom, A. (1994) Bacteria - Organic-Matter Coupling and Its Significance for Oceanic Carbon Cycling. *Microbial Ecology* **28**: 167-179.

Brown, B.E., and Bythell, J.C. (2005) Perspectives on mucus secretion in reef corals. *Marine Ecology-Progress Series* **296**: 291-309.

Clements, K.D., and Raubenheimer, D. (2006) Feeding and Nutrition. In *The Physiology of Fishes. Taylor and Francis Group, Boca Raton, FL, USA*. Evans, D.H., and Claiborne, J.B. (eds).

Garren, M., Smriga, S., and Azam, F. (2008) Gradients of coastal fish farm effluents and their effect on coral reef microbes. *Environmental Microbiology* **10**: 2299-2312.

Houlbreque, F., Tambutte, E., Richard, C., and Ferrier-Pages, C. (2004) Importance of a micro-diet for scleractinian corals. *Marine Ecology-Progress Series* **282**: 151-160.

Meyer, J.L., and Schultz, E.T. (1985) Migrating haemulid fishes as a source of nutrients and organic matter on coral reefs. *Limnology and Oceanography* **30**: 146-156.

Pinnegar, J.K., and Polunin, N.V.C. (2006) Planktivorous damselfish support significant nitrogen and phosphorus fluxes to Mediterranean reefs. *Marine Biology* **148**: 1089-1099.

Wild, C., Rasheed, M., Werner, U., Franke, U., Johnstone, R., and Huettel, M. (2004) Degradation and mineralization of coral mucus in reef environments. *Marine Ecology-Progress Series* **267**: 159-171.

Yahel, G., Sharp, J.H., Marie, D., Hase, C., and Genin, A. (2003) In situ feeding and element removal in the symbiont-bearing sponge Theonella swinhoei: Bulk DOC is the major source for carbon. *Limnology and Oceanography* **48**: 141-149.

Yahel, G., Marie, D., Beninger, P.G., Eckstein, S., and Genin, A. (2009) In situ evidence for pre-capture qualitative selection in the tropical bivalve Lithophaga simplex. *Aquatic Biology* **6**: 235-246.

CHAPTER 5: Effects of parrotfish feces deposition on corals and coral-associated microbial assemblages

ABSTRACT

Coral reefs at Palmyra Atoll harbor relatively high herbivorous fish biomass and consequently corals may experience a persistent downward flux of feces. At a Palmyra backreef site characterized by high coral cover, fecal pellet mass for individual parrotfish (*Chlorurus sordidus*) was positively correlated to fish length, and *C. sordidus* feces flux was estimated at 633 g dry wt m^{-2} yr^{-1}. In response to naturally-occurring feces settlement onto their surfaces, whole coral colonies of *Pocillopora verrucosa* and *Montipora aquituberculata* produced relatively low quantities of mucus while *Acropora hyacinthus* produced copious amounts that enveloped fecal particles into 'bundles.' Apparent cell-specific aminopeptidase activity was higher in *Acropora*-derived bundles than in feces, which suggests that feces settlement can stimulate the release of coral mucus-associated hydrolytic enzymes into the environment. To simulate feces settlement artificially, feces were transplanted atop *Montipora* surfaces and mucus and bundles were collected over 1 h. Microbial community composition associated with *Montipora*-derived bundles, as assessed via denaturing gradient gel electrophoresis (DGGE), appeared to be dominated by fecal phylotypes but also contained some mucus phylotypes. In addition to *in situ* observations, three aquaria-based experiments were performed where coral fragments were challenged with acute doses of aged parrotfish feces. *Montipora* and *Pocillopora* coral fragments that were treated with feces displayed no coloration changes over 92 h in aquaria, and similarly, *Acropora* fragments displayed no changes over 115 h.

Microbial assemblages associated with untreated *Montipora* coral fragments appeared to be the same as feces-treated fragments, while assemblages associated with both untreated and feces-treated *Acropora* fragments showed no consistent community profile among several replicates. Meanwhile, microbial assemblage composition associated with fecal particles that accumulated in the bottom of aquaria did not appear to be effected by the presence of coral fragments. In aquaria seawater, bacteria abundance increases over 17 h in feces-amended aquaria were higher than abundance increases in non-amended aquaria, but the abundances did not increase in aquaria that housed *Acropora* coral fragments. Meanwhile, C:N ratios of fecal particles that accumulated in aquaria were 23.7 (±3.9) in 'feces only' aquaria and 31.0 (±3.9) in feces-treated aquaria that housed *Acropora* fragments. I discuss the implications for coral reefs like Palmyra that harbor robust fish assemblages.

INTRODUCTION

Organic aggregates in coral reefs may be a source of nutrients for pelagic reef organisms and provide a link between primary production from corals and food webs within reefs and adjacent coastal oceans (Marshall, 1968); (Johannes, 1967). Coral mucus can be released into the water column as 'sheets' or 'fluid' or 'flocs' depending on coral species, physiological state, sunlight, depth, morphology, diurnal/annual cycles, and other factors (Coffroth, 1990). The released mucus can create aggregates which then entrain pelagic particles and have potential to sink into benthic sediments where they may become remineralized (Wild*, et al.*, 2004); (Wild*, et al.*, 2005). Coral

mucus secretion may be a protection mechanism against sedimentation or highly turbid waters (Hubbard & Pocock, 1972, Schuhmacher, 1977, Rublee*, et al.*, 1980, Riegl & Branch, 1995, Telesnicki & Goldberg, 1995, Gleason, 1998).

Fish feces, like sediments and other particles, may stimulate coral mucus production. Feces from parrotfish and other Scarids are not frequently consumed by coprophagous fishes (Robertson, 1982). They have high ash content due to carbonate particles from ingested coral and algal skeletons and are of low caloric value relative to feces from fishes in other trophic levels (Bailey & Robertson, 1982). Upon egestion, many parrotfish fecal pellets disintegrate in the water column as they sink and settle onto benthic surfaces including living corals. In their potential for smothering corals, parrotfish feces are similar to terrestrial sediments. Feces from parrotfish (*Chlorurus sordidus*) and other species contain highly abundant microbial assemblages that have potential for rapid growth, and feces from some fishes are associated with taxonomically distinct vibrio-like bacteria (Chapter III).

This study explores the phenomenon of feces-stimulated production of coral mucus, the tolerance limits of corals to feces deposition, and the potential ecological roles for bacteria within these processes. An overarching hypothesis is that fish feces influences coral health including changes to coral-associated microbial communities. The specific hypothesis addressed here is that acute accumulation of parrotfish feces is followed by qualitative and quantitative responses in coral physiology as well as shifts in the microbial community compositions associated with corals and feces.

I qualitatively describe the responses of three coral species to fish feces deposition atop their surfaces and the subsequent creation of 'feces-mucus-bundles' from one coral species. I describe artificial production of 'feces-mucus-bundles' via experimental dosing of live corals with parrotfish feces. This was done both *in situ* on whole coral colonies and in aquaria experiments on coral colony fragments. Additionally, I describe coral responses to feces challenges in aquaria experiments and potential responses of microbial assemblages on corals, feces, and water in the aquaria.

METHODS

All samples were collected and field observations made during September and October 2008 at ~5 m depth at sites in the Western Reef Terrace at Palmyra Atoll in the central Pacific Ocean (5°53'N 162°05W).

Collection of individual parrotfish feces and estimation of feces flux

Feces from 25 individual *Chlorurus sordidus* were collected immediately upon egestion using 10-mL syringes while SCUBA diving. Care was taken to exclude benthic sediments and other particles on which feces settled. Fish length and percentage of feces recovered were visually estimated. Fecal pellets were formaldehyde-fixed and stored at -20°C for ~2 months. The pellet suspensions were then thawed, collected onto preweighed 25 mm GF/F membranes (Whatman), and dry

weight determined. In addition to mass and flux, feces settlement sites (i.e. live coral, sediments, etc) were documented for ~30 separate feces egestion events.

Feces flux for *C. sordidus* at the Western Reef Terrace was estimated using empirical data, i.e. the size-specific fish abundances and defecation frequencies for *C. sordidus* that were observed by teams of SCUBA divers, and the dry weights that were determined from collected individual fecal pellets. Size-class-specific mean densities (m^{-2}) for *C. sordidus* were: 0.13 (0 - 5 cm), 0.0678 (5 - 10 cm), 0.0152 (10 – 15 cm), 0.0163 (15 – 20 cm), 0.0272 (20 – 25 cm), 0.0073 (25 - 30 cm), and 0.0008 (30 – 35 cm) (Sandin et al., unpublished data). Mean defecation frequency was 0.48 min^{-1} among fish of all sizes; there was no significant correlation between defecation frequency and size. The size-class-specific flux was calculated as the product of xyz, where x is the size-class-specific abundance of fish (m^{-2}), y is the defecation frequency (pellets $fish^{-1}$ h^{-1}), and z is the mean size-class-specific feces mass (mg dry weight $(pellet)^{-1}$). The last of these was determined from linear regression (Fig. 1), except for the smallest fish (0 – 5 cm) which used direct average. Additionally, to calculate annual flux it was assumed that fish defecated only 12 h d^{-1}, i.e. during daylight hours.

In situ observations of coral response to feces settlement and production of 'feces-mucus-bundles'

Observations of coral responses to 'naturally-occurring' feces settlement were made while SCUBA diving. Plating colonies of *Acropora hyacinthus*, encrusting colonies of *Montipora aquituberculata*, and branching *Pocillopora verrucosa* were

observed. Artificial feces dosing was performed and responses observed for *Acropora* and *Montipora*.

Aminopeptidase Activity in Aged Feces-Mucus-Bundles

To estimate aminopeptidase activity, aged parrotfish feces and aged feces-mucus-bundles from plating *A. hyacinthus* were collected using syringes and stored in ambient seawater ~3 h before assaying. The aged feces samples were then diluted in seawater collected at the same site. The feces-mucus-bundles were shaken vigorously before distribution into assay tubes. Activity was determined using the fluorogenic substrate leucine-7-amino-4-methylcoumarin (leu-AMC), which was added to 100 nM and incubated at 22°C in the dark for ~1 h (Hoppe, 1983). Five replicate samples were assayed. To control for possible adsorption of substrate onto feces particles, duplicate feces aliquots were sterilized via microwave for 5 min before dilution into assays; results for 'sterile' feces were subtracted from live assays. Fluorescence was determined with a fluorometer (Hoefer TKO-100) calibrated with AMC. Mass specific rates (i.e. mmol substrate (g dry wt feces) $^{-1}$ h^{-1}) were calculated, and cell specific rates (i.e. amol substrate cell^{-1} h^{-1}) were estimated from mass specific rates and the mean total cell abundances.

Field sample collection and preservation

The following sample types were collected from Western Reef Terrace: a) freshly egested feces from individual *C. sordidus* parrotfish ('feces')(described

above); b) settled particulate feces (‘aged feces’) that was accumulated in piles at dumping stations under plating corals or on dead calcareous reef ‘cement’; c) settled particulate feces that was accumulated naturally on live coral surfaces (‘aged feces-mucus-bundles’); d) coral mucus from colonies that were artificially dosed with aged feces (‘mucus’); and e) freshly produced feces-mucus-bundles resulting from artificial dosing (‘fresh feces-mucus-bundles’). Samples were collected with syringes that were previously unused or had been rinsed three times with distilled fresh water. The syringes with samples were stored in ambient seawater until processed, i.e. during transport back to the research station. All samples used for DNA extraction (DGGE) were preserved in RNA Later (Ambion) and stored at -20°C. Some samples (aged feces, feces-mucus-bundles) were kept live for aminopeptidase assay and aquaria experiments before preservation with formaldehyde.

PCR amplification of 16S rRNA genes

DNA was extracted from preserved samples using the UltraClean Soil Kit (MoBio). PCR amplification of 16S rRNA genes for DGGE analyses used the same primers and reaction conditions as previously described (Smriga*, et al.*, 2010). However, instead of a nested approach, a 35-cycle touchdown amplification program was used where the annealing temperature started at 65°C for the first cycle and was reduced 0.5°C per cycle to 50°C (Muyzer*, et al.*, 1993).

Aquaria experiments testing coral tolerance to feces deposition

Three aquaria-based experiments were performed sequentially over 17 days at the Palmyra research station. The purpose was to test the tolerance limits of corals to feces deposition. The coral species chosen for the tests had high proportional occurrence at Western Reef Terrace (WRT). For each experiment, coral fragments were collected from the WRT and were acclimated in 1 L plastic aquaria for two days prior to the start of the experiment. Each aquarium (24 total) was equipped with an airstone and continuous airflow was provided by hobbyist-grade aquaria airpumps (Fig. 3). Half volume of seawater was replaced in all aquaria approximately twice daily throughout the experiment with freshly collected WRT water (Fig. 3). To regulate temperature, aquaria were kept submerged in a 1 x 1 m bin filled with seawater that was placed under an awning that restricted access by direct sunlight, and ice was added to the bin water during afternoon hours. Aquaria temperatures over 17 d ranged 24.9°C to 28.7°C. The specific design for each experiment is described below.

Experiment 1:

Fragments were collected from four separate colonies each of *Montipora aquituberculata* and of *Pocillopora verrucosa*. The coral fragments were further partitioned into two smaller fragments, thus four clonal replicate fragments were treated with feces and four fragments were untreated controls. Parrotfish feces 'doses' were prepared daily. To do this, aged feces were collected during morning dives (~10:00 to 12:00) and diluted to 10% (v/v) using reef seawater. Ten mL of the feces

slurry was dosed into each 1 L aquarium just below the water surface, which created turbid conditions for several hours as the fecal particles settled. Dosing was additive in that particles from the prior dose were not removed from aquaria before the next addition. Four fragments for each coral species were dosed once daily with feces while four control fragments were not. The experiment began with the first feces dose after the acclimation period and was maintained for ~92 h (Fig. 3).

Experiment 2:

Coral fragments of *Acropora hyacinthus* were collected and acclimated in aquaria as above. Like experiment 1, feces doses were applied once daily to four clonal fragments (see Fig. 3). Additionally four aquaria that did not contain coral fragments were dosed with feces, and another four control aquaria were not dosed at all. Hence, the treatments for this experiment were: a) control corals; b) corals dosed with feces; c) control seawater; and d) seawater dosed with feces. The experiment was maintained for 96 h (Fig. 3).

Experiment 3:

A. hyacinthus coral fragments were collected and acclimated as in the prior two experiments. The treatments for this experiment were: a) control corals; b) corals dosed with feces for the entire experiment duration; c) corals dosed with feces for only two days and then allowed a 'recovery' period until the end of the experiment; and d) corals dosed with feces for only one day and then preserved. In contrast to Experiments 1 and 2, feces doses were applied twice daily. For the 'recovery'

treatment, settled fecal particles were removed 1 d after the dosing. The experiment was maintained for 115 h (Fig. 3).

Preservation of aquaria coral blastates, fecal particles, and seawater

At the end of each experiment, an airbrush was used to create 'blastate' comprised of mucus and tissue from ~2.5 cm^2 area of each coral fragment. Filter-sterilized seawater was used in the airbrush and mucus-tissue slurry volumes ranged 5 to 10 mL. An aliquot of each slurry (1 mL) was fixed with formaldehyde to 2% final concentration and frozen immediately at -20°C, while a separate aliquot (1.5 mL) was frozen unamended.

Fecal particles that settled during the experiments were also preserved in 2% formaldehyde at the end of each experiment (additionally at 5 and 22 h for Experiment 2). They were collected using sterile 10 mL pipets and thus the slurries contained entrained aquaria seawater. Aged feces used for dosing were also formaldehyde-fixed for all dosing timepoints in Experiments 2 and 3. Additionally, aquarium seawater (10 mL) was preserved with formaldehyde during Experiment 2 (5, 22, and 96 h timepoints); the water was preserved before coral fragments or fecal particles were removed in order to avoid resuspended particles.

RESULTS

In situ observations at Western Terrace Reef, Palmyra Atoll

a) Feces flux and settlement sites for individual parrotfish feces

Individual fecal pellet mass from Chlorurus sordidus correlated positively with fish length (Fig. 1). Mean size-class-specific feces dry weight ranged 1.8 to 119 mg pellet^{-1} (Fig. 1), while the mean for individuals of all lengths was 28.5 (±9.4) mg pellet^{-1}. The sum flux for all fish size classes was 144.9 mg m^{-2} h^{-1} or 632.8 g m^{-2} yr^{-1}, however flux varied by size class (Fig. 1). In observations of other egestion events, fecal pellets settled onto several benthic types including: stony corals (15 of 30 observed egestion events, or 50%), mixed substrate (whereby the fecal pellet fractured and settled onto multiple benthic types; 6 of 30 egestion events, or 20%), dead 'coral rubble' covered with crustose coralline algae (17%), sand (10%), and corallimorph (3%). The distribution of settlement sites roughly mirrored benthic coverage at the Western Terrace reef sites, which were dominated by live corals and crustose-coralline-algae-covered substrate (data not presented).

b) In situ coral responses to feces settlement and production of 'feces-mucus-bundles'

Coral behavior in response to naturally-occurring settlement of parrotfish feces were observed qualitatively for *Pocillopora verrucosa*. Within seconds of egestion from *C. sordidus*, the feces generally settled as patches of scattered particles and dispersed among the branches of the coral structure. If the pellet landed on a coral, the particles began to aggregate within minutes and small amounts of coral mucus (<1

mm^3) were incorporated. The fecal aggregates migrated a few centimeters laterally and downward toward the base of the colony. Within ~10 min, the feces-mucus aggregates disintegrated into smaller pieces. Some of the smallest were released into the water column and moved slowly (~1 m min^{-1}) until coming into contact with other surfaces. The larger aggregates remained associated with the *Pocillopora* colony and continued to migrate downward over minutes until they fell through the branches and onto surfaces that included dead coral 'cement' and other living corals.

For *Montipora aquituberculata*, no naturally-occurring settlement events were observed. However, I observed 'aged' aggregates that had already settled onto living colonies for an unknown amount of time. The fecal pellets were at various stages of disintegration, and small amounts of mucus were observed around the margins of the settled particles. Fecal aggregates did not move nor appear to disintegrate over ~30 min.

Naturally-occurring settlement events were also not observed for plating *Acropora hyacinthus*. However, in contrast to *Montipora*, 'aged' settled fecal pellets could be observed at what appeared to be various stages of disintegration. Some feces particles (<1 mm diameter) were entrained within fine wispy strands of coral mucus. Larger fecal aggregates were settled deeper into the coral skeletal matrix and were also enveloped in thicker mucus strands. Upon disturbance by gentle wafting, small aggregates (~1 to 3 cm) containing entrained feces became suspended in the water column. I refer to these aggregates as 'feces-mucus-bundles'. Some 'bundles' were more buoyant and became entrained in local water currents. Other 'bundles,'

particularly those with larger fecal particles, sank relatively quickly or became entangled in nearby coral branches.

To simulate naturally-occurring settlement events, feces particles were artificially dosed onto the surface of one *M. aquituberculata* colony and one plating *A. hyacinthus* colonies. In both cases the fecal particles immediately disintegrated into aggregates of various sizes as they settled. For the *Montipora* colonies, no visible changes could be observed within 60 min of dosing. For the *Acropora* colonies, within 5 min mucus strands were not visible. Within 30 min, small amounts of mucus were observed around the largest aggregates. Within 60 min, mucus strands were more pronounced around the large aggregates and the small aggregates were also becoming enveloped in mucus strands. However, the settlement sites did not yet exhibit the milky appearance of 'aged' aggregates described above and there was no release of 'bundles.'

c) Aminopeptidase activity in naturally-occurring 'aged' fecal aggregates

Mean bulk aminopeptidase activities were 33 (±9.3) nmol h^{-1} for seawater, 4718 (±627) nmol h^{-1} for aged feces, and 363 (±171) nmol h^{-1} for aged feces-mucus-bundles from *A. hyacinthus* corals (n =5 for all sample types). Mean cell abundances in assay tubes were 2.08 x 10^5 (±4.07 x 10^4) for seawater, 1.57 x 10^7 (±3.00 x 10^6) for aged feces, and 8.48 x 10^5 (±4.91 x 10^5) for aged feces-mucus-bundles. Mean cell-specific aminopeptidase activities were 168 (±30) amol leucine-AMC $cell^{-1}$ h^{-1} for seawater, 322 (±42) for aged feces, and 723 (±313) for aged feces-mucus-bundles.

The corrected mean activity (i.e. seawater subtracted) was significantly higher (3.6-fold) in aged feces-mucus-bundles than in aged feces.

d) Bacterial assemblage composition associated with in situ artificial feces settlement

DGGE profiles of microbial assemblages from *Montipora* mucus (Fig. 2, lanes 1-6) were distinct from profiles of feces (lanes 7-8) or fresh feces-mucus-bundles (lanes 9-10). Profiles for fresh feces-mucus-bundles were more similar to feces than to mucus, as exemplified by the occurrence of a high GC band near the bottom of the gel (B and D). There were no obvious changes in assemblage composition within mucus over the short time course of 1 h. Nonetheless, there were bands that co-migrated among the different sample types. Band A occurred in all samples (Fig. 2). Bands B and C occurred in all of the mucus samples (lanes 1-6) and in the fresh feces-mucus-bundles t5 sample (lane 9), but not in the feces samples (lanes 7-8). Similarly, Band D occurred in the feces and the feces-mucus-bundle samples but not in the mucus samples (Fig. 2).

Aquaria experiments testing coral tolerance to feces deposition

a) Coral fragment responses to feces challenges in aquaria

Feces-treated *Montipora* and *Pocillopora* coral fragments displayed no qualitative physiological changes over 92 h in aquaria (see Methods, Experiment 1). Feces-treated corals appeared no different than control corals. Coloration remained constant and no lesions or spots developed. Generally within ~12 h of each dosing

with aged feces, the corals had removed fecal particles that settled on their surfaces. Similarly, *Acropora* coral fragments displayed no qualitative changes in response to feces challenges over 96 h (Experiment 2) or 115 h (Experiments 3). Furthermore, *Acropora* coral photosynthetic efficiency did not significantly differ between control corals and feces-treated corals after 115 h (Experiment 3 only; data not presented).

b) Coral-associated assemblage composition in aquaria evaluated via DGGE

DGGE profiles for microbial assemblages from control *Montipora* coral fragments were similar to those from feces-treated fragments (Fig. 4). Triplicate clonal fragments displayed relatively similar profiles, and fragments from one colony (M-3) displayed a distinct low-GC band in control and feces treatments (Fig. 4).

Conversely, DGGE profiles for assemblages associated with *Acropora* coral fragments appeared dissimilar (Fig. 5). There was no similarity in banding patterns among four replicate control fragments (A1C thru A4C) nor among four replicate feces-treated fragments (A1F thru A4F). Also, clonal pairwise profiles were dissimilar, i.e. the profile for fragment 1 control (A1C) was dissimilar to the profile for fragment 1 feces-treated (A1F). However, pairwise profiles were similar for PCR amplicons resulting from different amplification programs, i.e. the profile for fragment 3 control via touchdown PCR (A3C, TD-PCR) was the same as the profile for fragment 3 control via standard PCR (A3C, PCR)(Fig. 5).

c) Feces-associated assemblage composition in aquaria evaluated via DGGE

DGGE profiles for assemblages associated with settled feces particles were similar between control aquaria ('feces only') and those that housed *Acropora* corals (feces + coral)(Fig. 6). Meanwhile, profiles from settled particles in two 'coral only' control aquaria (A1C-P and A2C-P, which assumedly contained settled mucus aggregates and dead plankton) were dissimilar. They also were distinct from the profiles of their clonal replicates in 'feces + coral' aquaria (Fig. 6, A1F-P and A2F-P). However, some bands between the two sample types co-migrated.

d) Carbon and nitrogen composition of feces particles in aquaria (Experiment 2)

C:N ratios were determined for aged feces particles that were used for dosing (which were collected over different days) as well as for feces particles that settled in the bottom of aquaria during Experiment 2. Mean C:N ratios (±S.E.) were 37.2 (±4.6)(n = 4) for aged feces, 23.7 (±3.9)(n = 3) for 'feces only' control aquaria, and 31.0 (±3.9)(n = 4) for 'feces + coral' aquaria that housed *Acropora* fragments. The last of these may be analogous to 'feces-mucus-bundles' since they may include mucus released by *Acropora* fragments.

e) Bacterial abundances in seawater and settled fecal particles in aquaria (Expt. 2)

Bacteria abundances in aquaria seawater changed over hours and the changes depended on the presence or absence of feces and corals (Fig. 7). In the absence of corals, seawater bacteria abundances were higher in feces-treated aquaria (+feces) than

in non-treated aquaria (control) 5 h after amendment with feces (the first timepoint for sampling). Furthermore, 5 h after amendment, abundances were higher in aquaria that contained corals ('+feces +*Acropora*' and 'control +*Acropora*') than in those that did not ('+feces' and 'control'). Over 17 h incubation, seawater bacteria abundances increased 4.1-fold in feces-treated aquaria while they increased 4.4-fold in non-treated control aquaria. In contrast, bacteria abundances decreased over 17 h in parallel treatments that contained *Acropora* coral fragments (Fig. 7).

Similarly, the presence of corals affected bacteria abundances associated with fecal particles that settled in aquaria. Mean abundance (±S.E.) for settled particles in feces-treated aquaria was 6.2 (±0.48) x 10^9 g dry wt^{-1} (n = 3) while for feces-treated aquaria that housed *Acropora* it was 22 (±4.8) x 10^9 g dry wt^{-1} (n = 3). In other words, bacteria abundances in settled feces particles were 3.6-fold higher in the presence of *Acropora* coral fragments after 96 h. By comparison, mean abundances in aged feces used for dosing was 5.6 (±0.43) x 10^9 g dry wt^{-1} (n = 3).

DISCUSSION

It has long been recognized that corals produce mucus in response to particle settlement on their surfaces (e.g. (Hubbard & Pocock, 1972)). The present study is the first to describe parrotfish feces as a particle type that can induce mucus production. The responses differed among the three coral species for which this process was observed at Palmyra Atoll. Plating *Acropora hyacinthus* produced copious mucus

within minutes of feces settlement. *Pocillopora verrucosa* appeared to produce less mucus but were able to quickly shunt fecal particles off of the colony. Meanwhile, *Montipora aquituberculata* produced small amounts of mucus but showed little ability to remove particles within minutes to hours. The latter observation suggests this species of *Montipora* is adapted to cope with periodic smothering by feces and other particles. These differing responses by corals suggest that feces-associated bacteria will interact with living coral surfaces under varying conditions.

It was remarkable that all three coral species displayed no qualitative responses to acute challenges with parrotfish feces in aquaria experiments. Similar results were observed during a separate field experiment that ran concurrently at Palmyra Atoll (Sandin, unpublished data). In the experiment, ~10 tagged colonies on the reef were challenged daily over 25 d with sediments or parrotfish feces. The corals' coloration did not change and they appeared to remain healthy. Our collective observations for these coral species at Palmyra Atoll do not corroborate with the paradigm that sedimentation causes coral stress which can lead to loss of live coral cover. However, all three experiments were performed over less than one month and this relatively short time scale probably only tested acute and not chronic sedimentation.

The individual feces production rates (ranging 0.16 to 10.9 kg ind^{-1} yr^{-1}; Fig. 1) and total feces flux by *C. sordidus* (632.8 g m^{-2} yr^{-1}; Fig. 1) provide constraints on the contributions of parrotfish to the transport of biomass at Palmyra. A previous study (Bellwood, 1996) estimated bioerosion rates of 24 kg ind^{-1} yr^{-1} for *C. sordidus* at two sites in the northern Great Barrier Reef. In contrast to the present study that measured

dry weight mass of egested feces, Bellwood (1996) estimated ingestion of carbonate substrate by measuring bite size volume and substrata density, among other parameters. The study found differences by geographical site (scale of meters) as well as daily and seasonal differences in *C. sordidus* feeding rates, all of which have yet to be considered at Palmyra and may account for the apparently lower individual feces production rates. Regardless, the flux at Palmyra represents a potential major contribution of particles to coral surfaces. Given ~1 x 10^9 bacteria g^{-1} dry wt feces from *C. sordidus* (Chapter III), the downward flux of feces-associated bacteria would be ~0.1 x 10^6 cells cm^{-2} d^{-1}. If the surface area of coral mucus contains ~1 x 10^6 bacteria cm^{-2}, then the daily addition of fecal bacteria is ~10% of total cell abundance in the mucus. Dynamic processes likely occur, e.g. there may be diel cycles in mucus-associated cell abundances. However, coral-driven mechanisms that maintain near steady-state abundances must be in effect, or else fecal bacteria would overwhelm a coral surface over just days.

The placement of feces onto corals in order to artificially stimulate mucus production provided a model for 'natural' production of mucus and feces-mucus-bundles. My approach was and it is unclear how accurately artificial stimulation simulated natural processes. However, a simple technique was used previously to simulate the production of pelagic marine snow (Shanks & Edmondson, 1989), and these model aggregates have been essential for exploring fundamental interactions among microbes and particles in the surface ocean. Mucus production by the corals tested here (*Acropora* and *Montipora*) is most likely a generalized defense mechanism

for the removal of settled particles (Brown & Bythell, 2005). Ciliary action on coral surfaces is another mechanism that aids in removal of particles (Brown & Bythell, 2005). These generalized responses prevent smothering but may serendipitously remove potential microbial pathogens associated with feces and other particles. Given that feces-associated bacteria can experience rapid growth (Smriga et al., *in press*), removal of feces may also prevent localized accumulation of growing bacteria or 'recruitment' of seawater bacteria toward feces on the coral surface.

The 16S rRNA DGGE profiles for the various *in situ* and aquaria experiments demonstrated that bacterial assemblages associated with *Montipora* corals were relatively distinct from assemblages associated with parrotfish feces. Only a few DGGE bands from coral mucus co-migrated with bands in feces-mucus-bundles (e.g. Fig. 2, lane 8). Nonetheless, these results support the hypothesis that some mucus phylotypes have potential to become entrained into feces-mucus-bundles and interact with feces-associated bacteria. The highly dissimilar DGGE profiles for replicate *Acropora* fragments (Fig. 5) limits our ability to make conclusions regarding these bacterial assemblages. The dissimilarities likely did not result from PCR amplification artifacts because profiles generated from two different PCR amplifications (standard and touchdown) were highly similar. Instead, the dissimilarities may have resulted from multiple possible scenarios. One possibility is that highly dissimilar phylotypes dominated each clonal coral fragment, i.e. the variance among associated microbial assemblages was so high that co-migration of DGGE bands did not occur. If this was the case, then the low band number observed

in each profile suggests very low diversity existed in each fragment. A second possibility is that bacterial abundances associated with *Acropora* were so low that PCR amplicons were representative of rare phylotypes that spuriously 'dominated' each clonal fragment. Even if this *Acropora* species at Palmyra hosted low bacterial abundances, it does not preclude that the same species at other coral reefs hosts abundant assemblages. An ancillary to this scenario is that a well-established bacterial assemblage was present throughout the *Acropora* colonies, but the cell abundances were so low that DNA from spurious contaminating seawater bacteria were stoichiometrically favored by primers in the PCR reaction. A third possibility is that heterogeneously distributed chemical compounds inhibited PCR amplification for some phylotypes in some coral fragments but not others.

With regard to fecal bacteria, this study focused on potential changes in community composition, but it is also important to consider their potential ecological roles. They may express hydrolytic enzymes to degrade algal POM then incorporate the dissolved algal OM and convert it into bacterial biomass. Indeed, apparent cell specific aminopeptidase activity in aged feces was nearly 2-fold higher than in seawater (see Results), which is consistent with high apparent activities previously observed in fresh feces (Smriga*, et al.*, 2010).

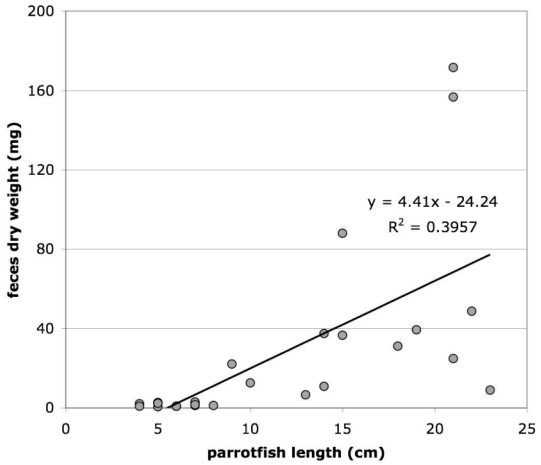
An important hypothesis to test is whether fecal enzymes that settle onto corals cause degradation of mucus or prevent mucus formation and cause stress to the coral. Fecal aminopeptidases may include those expressed endogenously by fish or other non-bacterial sources such as eukaryotic protists. Interestingly, apparent cell specific

aminopeptidase activity was ~3.6-fold higher in coral-mucus-bundles than in aged feces. This suggests that mucus-associated bacteria could be expressing high aminopeptidase activity, perhaps to utilize mucus glycoproteins. However, similar to feces, coral-derived aminopeptidases may have been entrained into mucus and contributed to the apparent activity. Future studies should estimate coupling between bacterial aminopeptidase activity and amino acid incorporation as a test for whether coral bacteria can utilize fecal OM.

Bacteria abundance increases were greater in aquaria seawater treated with parrotfish feces than in non-dosed seawater over a 17 h incubation (Fig. 7). This bacterial growth was likely supported by fecal DOM and POM, which is corroborated by the observation that 5 h after dosing (the first of two sampling timepoints) the feces-treated seawater already contained higher cell abundances than non-treated seawater (Fig. 7). Meanwhile, lower cell abundances in the presence of *Acropora* coral fragments (Fig. 7) may have been caused by coral filter feeding. Indeed, some reef waters contain lower bulk bacterial abundances directly over reefs than in waters adjacent to reefs (Seymour*, et al.*, 2005) and some corals ingest picoplanktonic prey (Houlbreque*, et al.*, 2004). Alternatively, corals may have exuded dissolved compounds that killed some seawater bacterial phylotypes and prevented the accumulation of others.

The results of this study are descriptive in nature due to logistical constraints but they begin to characterize the potential outcomes of feces settlement onto reefs. The corals tested here had high tolerance limits to feces deposition and some were

able to clear bulk material relatively quickly. The continual settlement of parrotfish feces at Palmyra may have acclimated these corals so that they can tolerate periods of acute sedimentation from feces or other sources. Meanwhile, the formation of feces-mucus-bundles may be an important ecological phenomenon. Bundles may behave as incubators whereby organically rich feces mixed with organically rich coral mucus enable 'culture-like' conditions for bacterial growth. They may act as vectors for transferring bacteria among colonies within a coral species or among colonies of multiple species. Furthermore, they may carry organic matter and associated bacteria between different reef strata, e.g. from forereef to backreef. and may be consumed by fish. Thus they may represent an additional trophic linkage between benthic organisms and fishes in reefs. The interaction between fish feces and corals represents an exciting horizon for testing hypotheses that address microbial contributions to biogeochemical dynamics.



Fish length (cm)	Feces mass (mg pellet^{-1})	Individual rate (kg ind^{-1} yr^{-1})	Feces flux (mg m^{-2} h^{-1})	(g m^{-2} yr^{-1})
0-5	1.8	0.16	6.7	29.5
5-10	8.84	0.81	17.3	75.6
10-15	30.9	2.83	13.5	59.2
15-20	52.9	4.85	24.9	108.9
20-25	75.0	6.87	58.8	257.6
25-30	97.0	8.89	20.5	89.6
30-35	119	10.9	2.9	12.5
Sum	**n/a**	**n/a**	**144.5**	**632.8**

Figure 5-1: Dry weights of individual feces (n = 25) from *Chlorurus sordidus* by fish length (graph) and mean size-class-specific feces mass and feces flux (table) for the Western Reef Terrace at Palmyra Atoll. Mean size-class-specific feces mass (table) was determined from linear regression (graph), except for the smallest fish (0 – 5 cm) which used direct average. Units for mass (mg and g) are dry weight. Feces flux is the estimated sum for all fish of a given size class.

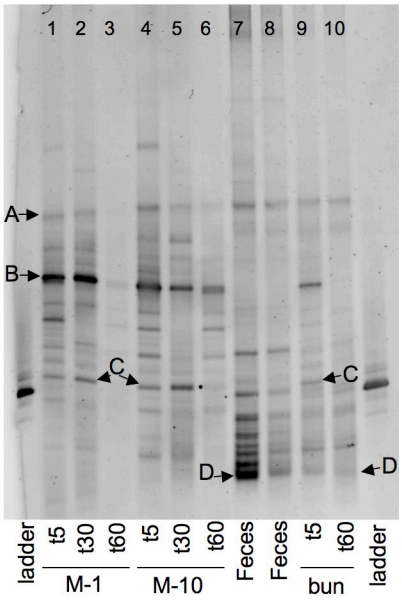


Figure 5-2: 16S rRNA gene DGGE profiles for coral mucus, aged parrotfish feces (Feces), and feces-mucus-bundles (bun) collected during short-term *in situ* incubations of feces dosed onto a *Montipora aquituberculata* coral colony. Coral mucus was collected ~1 cm (M-1) and ~10 cm (M-10) from the feces dosing site at 5, 30, and 60 min following the artificial dosing event (t5, t,30, and t60, respectively). Similarly, feces-mucus-bundles were collected 5 and 60 min after dosing. Band A appeared in nearly all sample profiles, Bands B and C appeared in all mucus profiles as well as 'bun t5', and Band D appeared only in 'Feces' and 'bun' samples.

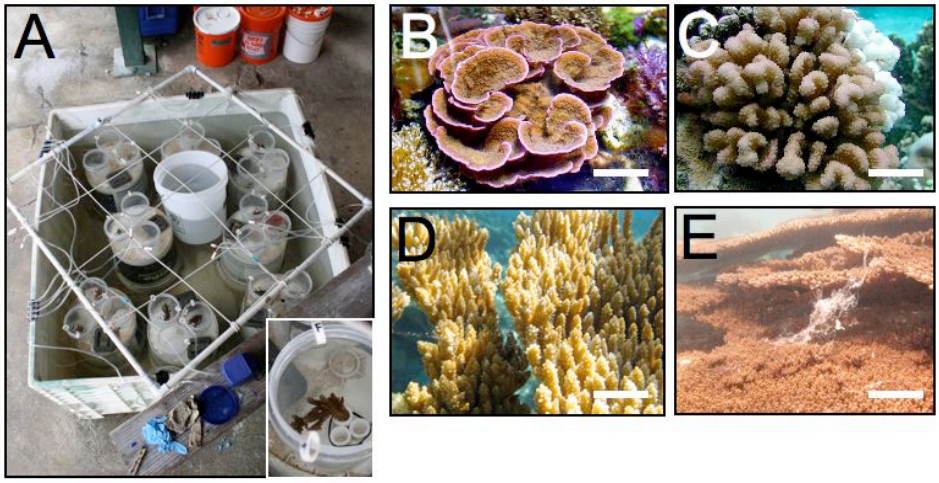


Figure 5-3: Design for aquaria experiments where coral fragments were treated with parrotfish feces. Panel A shows a water-filled bin (1 x 1 m) containing 24 aquaria (1 L) each equipped with an airstone. The inset shows a closeup of one aquarium housing an *Acropora* coral fragment. Also shown are images of a representative *Montipora aquituberculata* colony (Panel B; scale bar ~0.2 m), a representative *Pocillopora verrucosa* colony (Panel C; scale bar ~10 cm), and two *Acropora hyacinthus* colonies at Western Reef Terrace (Panel D; scale bar ~2 cm; and Panel E, scale bar ~20 cm). The *Acropora* colony in panel E is excreting copious coral mucus. Panel A (next page) illustrate timelines for manipulations during the three multi-day experiments. Timepoints for seawater exchanges (SW) and those when fish feces were dosed into aquaria (F) are indicated. During Experiment 3, the 'recovery' corals received only four feces doses (F*) and settled particles in these aquaria were removed at 65 h.

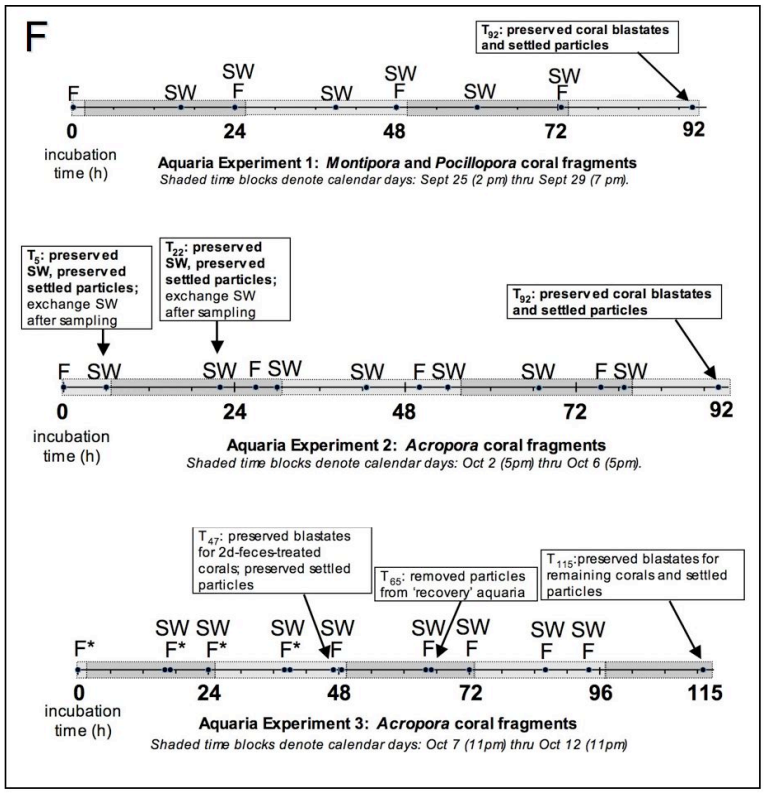


Figure 5-3: continued

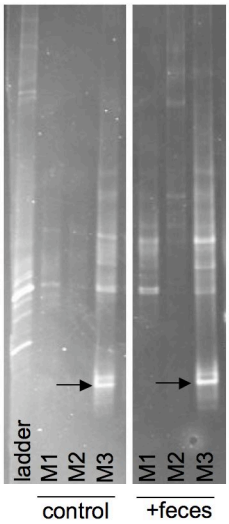


Figure 5-4: 16S rRNA gene DGGE profiles for blastates of *Montipora aquituberculata* coral fragments held in aquaria experiments for 92 h (Experiment 1). Aquaria were left unamended (control) or were amended daily with aged parrotfish feces (+feces). One fragment from each of three colonies (M1, M2, and M3) was tested per treatment. A distinct high-GC band was observed for M3 (arrow).

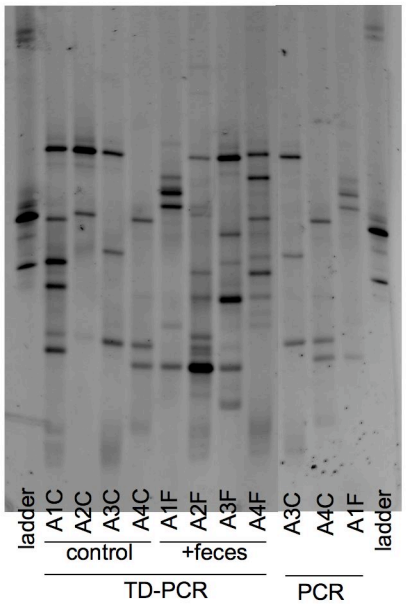


Figure 5-5: 16S rRNA gene DGGE profiles for blastates of *Acropora hyacinthus* coral fragments held in aquaria experiments for 96 h (Experiment 2). Aquaria were left unamended (control) or were amended once daily with parrotfish feces (+feces). One fragment from each of four colonies (A1 thru A4) was tested per treatment. Touchdown PCR (TD-PCR) was compared with standard PCR (PCR) for three blastates (A3C, A4C, A1F) for which there appeared to be no differences.

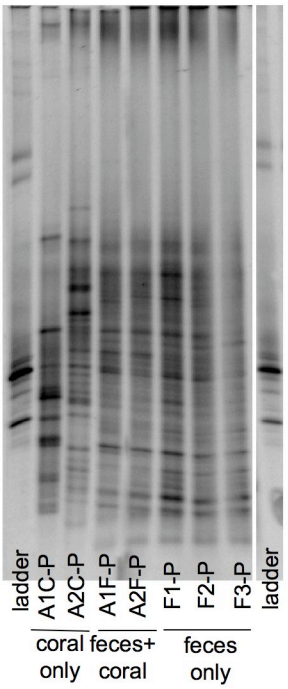


Figure 5-6: 16S rRNA gene DGGE profiles for bacterial assemblages associated with settled particles ('-P') accumulated in the bottom of aquaria over 96 h (Experiment 2). Control aquaria that housed *Acropora* fragments (coral only) contained low abundances of <1 mm particles. Feces-treated control aquaria (feces only) and those that housed *Acropora* coral fragments (feces+coral) contained abundant obvious fecal particles ranging up to ~2 mm in diameter. 'Feces only' and 'feces+coral' aquaria were amended once daily with aged feces.

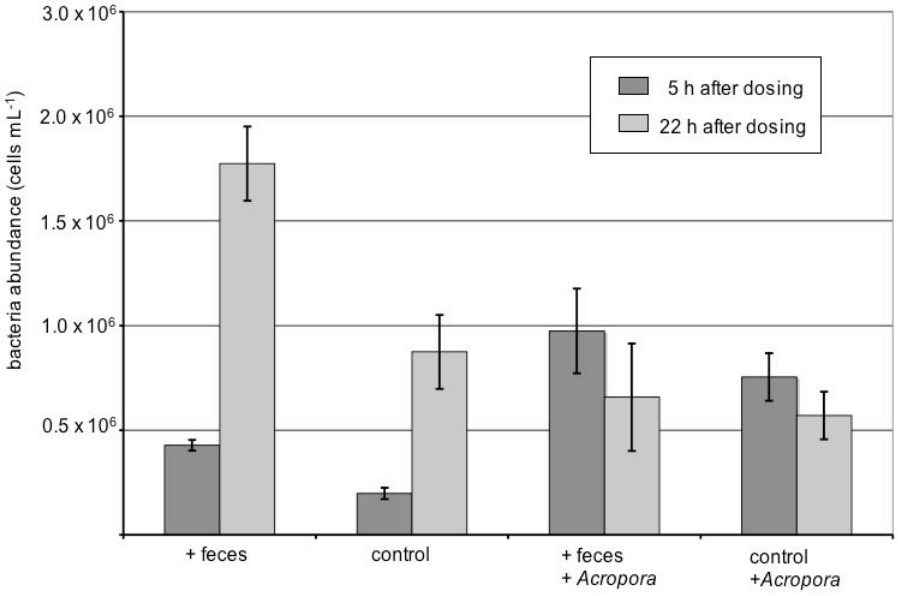


Figure 5-7: Seawater bacteria abundances in experimental aquaria. All aquaria contained ~1 L surface seawater collected from Western Reef Terrace, Palmyra Atoll. Those that were dosed with parrotfish feces are indicated (+feces) and those that housed coral fragments are indicated (+*Acropora*). Abundances increased over 17 h but not in the presence of *Acropora* corals. Error bars show ±S.E.

Acknowledgements

I thank S. Sandin and J. Smith as well as J. Baum, T. Davies, M. Garren, S. Hamilton, and N. Price for assistance with aquaria experiments, collection of fish observation data, and beneficial discussions during the 2008 Palmyra field trip. Support was provided by the GEF Coral Reef Targeted Research Program, a National Science Foundation grant (OCE0648116), The Gordon and Betty Moore Foundation, The Nature Conservancy at Palmyra Atoll, and an NSF Graduate Research Fellowship. Sample collection was permitted via the US Fish and Wildlife Service.

REFERENCES

Bailey TG & Robertson DR (1982) Organic and Caloric Levels of Fish Feces Relative to its Consumption by Coprophagous Reef Fishes. *Marine Biology* **69**: 45-50.

Brown BE & Bythell JC (2005) Perspectives on mucus secretion in reef corals. *Marine Ecology-Progress Series* **296**: 291-309.

Coffroth MA (1990) Mucous sheet formation on Poritid corals - An evaluation of coral mucus as a nutrient source on reefs. *Marine Biology* **105**: 39-49.

Gleason DF (1998) Sedimentation and distributions of green and brown morphs of the Caribbean coral *Porites astreoides* Lamarck. *Journal of Experimental Marine Biology and Ecology* **230**: 73-89.

Hoppe HG (1983) Significance of Exoenzymatic Activities in the Ecology of Brackish Water - Measurements by Means of Methylumbelliferyl-Substrates. *Marine Ecology-Progress Series* **11**: 299-308.

Houlbreque F, Tambutte E, Richard C & Ferrier-Pages C (2004) Importance of a micro-diet for scleractinian corals. *Marine Ecology-Progress Series* **282**: 151-160.

Hubbard JAEB & Pocock YP (1972) Sediment rejection by recent scleractinian corals: a key to palaeo-environmental reconstruction. *International Journal of Earth Sciences* **61**: 598-626.

Johannes RE (1967) Ecology of Organic Aggregates in the Vicinity of a Coral Reef. *Limnology and Oceanography* **12**: 189-195.

Marshall M (1968) Observations on organic aggregates in the vicinity of coral reefs. *Marine Biology* **2**: 50-&.

Muyzer G, Dewaal EC & Uitterlinden AG (1993) Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S Ribosomal RNA. *Applied and Environmental Microbiology* **59**: 695-700.

Riegl B & Branch GM (1995) Effects of sediment on the energy budgets of four Scleractinian (Bourn 1900) and five Alcyonacean (Lamouroux 1816) corals. *Journal of Experimental Marine Biology and Ecology* **186**: 259-275.

Robertson DR (1982) Fish Feces as Fish Food on a Pacific Coral Reef. *Marine Ecology-Progress Series* **7**: 253-265.

Rublee PA, Lasker HR, Gottfried M & Roman MR (1980) Production and bacterial colonization of mucus from the soft coral Briarium asbestinum. *Bulletin of Marine Science* **30**: 888-893.

Schuhmacher H (1977) Ability in fungiid corals to overcome sedimentation. *Proceedings of the Third International Coral Reef Symposium* **1**: 503-509.

Seymour JR, Seuront L & Mitchell JG (2005) Microscale and small-scale temporal dynamics of a coastal planktonic microbial community. *Marine Ecology-Progress Series* **300**: 21-37.

Shanks AL & Edmondson EW (1989) Laboratory-made artificial marine snow - A biological model of the real thing. *Marine Biology* **101**: 463-470.

Smriga S, Sandin SA & Azam F (2010) Abundance, diversity, and activity of microbial assemblages associated with coral reef fish guts and feces. *FEMS Microbiology Ecology* **73**: 31-42.

Telesnicki GJ & Goldberg WM (1995) Effects of turbidity on the photosynthesis and respiration of two South Florida reef coral species. *Bulletin of Marine Science* **57**: 527-539.

Wild C, Woyt H & Huettel M (2005) Influence of coral mucus on nutrient fluxes in carbonate sands. *Marine Ecology-Progress Series* **287**: 87-98.

Wild C, Huettel M, Klueter A, Kremb SG, Rasheed MYM & Jorgensen BB (2004) Coral mucus functions as an energy carrier and particle trap in the reef ecosystem. *Nature* **428**: 66-70.

CHAPTER 6: Use of ethynyldeoxyuridine incorporation and click chemistry to detect DNA synthesis in marine bacteria

ABSTRACT

We devised a protocol to detect growing bacteria in seawater using the thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU), which becomes incorporated into bacterial DNA during growth. The approach uses a 'click' chemistry reaction to detect incorporated EdU with an azide-modified fluorophore without the need for cell lysis. Bacterial growth in coastal seawater was not inhibited in 20 nM, 100 nM, or 1 μM EdU over a 7 h incubation. In a separate time-course incubation of coastal seawater, the percentage of EdU-labeled bacteria ranged 6.2% to 17.9%, and and the percentage of labeled cells determined via EdU was similar to the percentage determined via ^{3}H-thymidine microradiography. Meanwhile, four cultured marine isolates with different specific growth rates could incorporate EdU but incorporation did not occur in cells that were formaldehyde-fixed or were incubated on ice. EdU signals within individual cultured and natural assemblage cells displayed various distribution patterns. For example, some cells that contained two distinct EdU loci did not appear to be septated via the DNA-specific stain 4',6-diamidino-2-phenylindole (DAPI). Thus the method may be useful for labeling actively dividing cells as well as elucidating partitioning mechanisms of newly synthesized DNA in marine (and other) bacteria. The method can potentially be further developed to quantify individual cell growth rate. As an *in situ* approach for observing thymidine-incorporating cells, click chemistry should be complementary to the detection of incorporated ^{3}H-thymidine via microautoradiography or incorporated bromodeoxyuridine via immunochemistry. The EdU incorporation method may help constrain hypotheses on the individual-based

ecology of marine bacteria and their influence on ocean-basin scale biogeochemical processes.

INTRODUCTION

Marine Bacteria and Archaea ('bacteria') contribute to macroscale organic matter fluxes and elemental cycling in the ocean through microscale interactions (Azam & Malfatti, 2007). For example, bacteria assemblages associated with particles were found to express higher hydrolytic enzyme activities relative to 'free-living' bacteria, which enables the biogeochemically significant process of phase transition from particulate organic carbon to dissolved organic matter (Smith*, et al.*, 1992, Bidle & Azam, 2001). Our conceptual understanding of the role of microbial dynamics in regulating organic matter flux is continually strengthened by the development and use of techniques that detect and quantify individual-cell activities and growth at microscale resolution (del Giorgio & Gasol, 2008). Metabolic activities that can currently be detected in individual cells include respiration and esterase activity; and viable cells can be detected using live/dead stains, nalidixic acid, and observations of microcolonies on agar plates. These and other methods have revealed the potential for high variability in cell-specific metabolism within bacterioplankton assemblages (del Giorgio & Gasol, 2008).

A challenge to the field of microbial oceanography is quantification of individual bacteria cell growth rates by methods that are applicable in varied field settings. One approach is the detection of incorporated tritiated thymidine (^{3}H-TdR) via microautoradiography (Fuhrman & Azam, 1982, Douglas*, et al.*, 1987). While this

method has been used in microbial oceanography for several decades and has yielded important insights, its use is often limited by regulatory restrictions, especially in remote locations and other field settings. A non-radioisotopic alternative approach based on incorporation of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) was developed and tested on marine microbial assemblages. One study coupled BrdU immunochemical detection with fluorescence in situ hybridization (FISH) to detect the phylogenetic affiliation of growing cells in coastal seawater (Pernthaler*, et al.*, 2002). Another study optimized BrdU detection in marine bacteria and observed a positive correlation between growth rates and fluorescence intensity of growing cells (Hamasaki*, et al.*, 2004). Immunochemical detection of BrdU, as used in these studies, is a relatively low throughput technique that requires multiple processing steps including cell lysis, DNA denaturation, probing with anti-BrdU antibodies, and signal amplification via catalyzed reporter deposition.

Recently, a method that uses 5-ethynyl-2'-deoxyuridine (EdU) has been developed as an alternative to ^{3}H-TdR and BrdU in cell biology (Salic & Mitchison, 2008). Incorporated EdU is detected in cells by labeling with a fluorescent azide through a copper-catalyzed cyclo-addition, also called a 'click' reaction (Kolb*, et al.*, 2001). The relatively small sizes of molecules used in the reaction permit penetration through eukaryotic cell membranes and eliminate the need for DNA denaturation, antibody probing, or signal amplification.

Here we report the use of EdU to detect natural populations of DNA-synthesizing (‘growing’) bacteria in coastal seawater. We optimized a method for

microscopic detection and detail the parameters that were compared during this optimization. The dynamic range of single bacteria cell fluorescence intensity was quantified and compared to measurements of single-cell growth rates. We discuss the method's potential for single cell growth rate determination in marine microbes and for linking growth rates with microscale biogeochemical dynamics in the ocean.

MATERIALS AND METHODS

Filtration and lysis:

Three types of filter membranes were tested for collecting seawater bacteria: white polycarbonate (Millipore, GTTP02500), black polycarbonate (Millipore, GTBP02500), and Anodisc aluminum oxide filters (Whatman, 6809-6002). The diameter was 25 mm and the pore size was 0.2 μm for all membrane types. Seawater was filtered through membranes using a vacuum manifold, and volumes varied by experiment. After filtration, scissors or razor blades were used to remove one-quarter sized filter pieces for click detection reactions, and the remaining filter was stored at -20°C for future analyses.

Two cell permeabilization treatments were tested on filter membranes including lysozyme (50 mg mL^{-1} in TE buffer [10 mM Tris, 1 mM EDTA, pH 7.5], 30 min, 37°C) or pepsin (2 mg mL^{-1} in 0.01N HCl, 2 h, 37°C) followed by lysozyme (3 mg mL^{-1} in TE buffer, 15 min, room temp). Permeabilization treatments were tested based on the manufacturer's suggested protocol (Invitrogen) and a prior study that

demonstrated some eukaryotic cells require it prior to incubation with fluorescent azides (Salic & Mitchison, 2008).

Click chemistry reaction for detection of EdU:

All tests of EdU detection were performed using the Click-iT EdU AlexaFluor High-Throughput Imaging Assay kit (Invitrogen, Cat No. A10027). The kit includes EdU, AlexaFluor-488 azide, reaction buffer, $CuSO_4$, and reaction buffer additive. The 'buffer additive' component of the reaction mixture was added to the reaction cocktail just prior to use. Alexa Fluor-488 azide was used for the tests performed in this study, but other fluorophore azides are also available. Reagents were distributed and stored as per manufacturer's recommendations.

A one-quarter-sized sample filter piece was placed onto a clean glass slide. Glass cover slips (Corning, 25 x 25 mm, 2870-25) were washed with 70% ethanol and thoroughly dried. Reaction cocktail was prepared on ice per the manufacturer's instructions but volumes were adjusted accordingly depending on the number of filter pieces to be processed. For a one-quarter piece of 25 mm membrane, 25 μl reaction cocktail was sufficient and more cost effective. The cocktail (25 μl) was spotted onto the cover slip and inverted onto the filter on the slide. The slide was placed into an incubation chamber that consisted of a 50 mL disposable plastic tube (Falcon) containing a tissue paper (Kimwipe) soaked with TE buffer. The slide chamber was incubated in the dark for 30 min at room temp (~22°C). The filter pieces were then removed using forceps, dipped sequentially into two dishes of autoclaved water for ~2

s per bath, washed for 3 min. in autoclaved water in the dark, and then set to dry sample side up momentarily on tissue paper. To complete the drying, filters were mounted into office binder clips and placed in a 37°C oven for 3 - 4 min. Filters were then removed from the oven, placed onto a 10 µl drop of DAPI (1 ug mL^{-1}), incubated in the dark for 5 min at room temperature, and rinsed and dried as above.

In addition to the adopted 'cover-slip-inversion' method described above, three other methods for applying reaction cocktail were tested. One was the same as above except a sterile hydrophobic coverslip was used (Hybrislip Hybridization Covers, 22 x 22mm, No. 247455). A second technique used a 'pool' approach in which ~100 ul of reaction cocktail was added to the sample side of the filter piece on a glass slide. Surface tension kept the pool in a 'bubble' so that the liquid did not diffuse laterally. A third technique used 'filter perfusion' whereby 12 µl of reaction cocktail was spotted onto a glass slide and the filter piece was placed atop the cocktail with the sample side up. This is similar to a method for applying the nucleic acid stain Sybr Green I to enumerate virus particles (Noble & Fuhrman, 1998).

Slide preparation:

Mounting media tested in this study included VectaShield with DAPI (Vector Laboratories, H-1200) and a glycerol-based anti-fading formulation (1:1 glycerol and PBS, 0.1% w/v phenylenediamine; (Noble & Fuhrman, 1998). Ten µl of media were spotted onto the slide, the dry filter piece was placed sample-side-up atop the spot, and a coverslip containing another 10 µl of media was placed onto the filter piece.

Processed filters were viewed or imaged the same day that they were mounted to slides in order to observe optimal signal intensity.

Microscopy and image analysis:

Qualitative observations of slides were made using an Olympus BH51 epifluorescence microscope equipped with a UPlanApo 100x objective. Images for quantification were made using a Nikon Eclipse TE-2000 inverted epifluorescence microscope equipped with a 100x objective, a monochrome CoolSNAP HQCCD camera (Roper Scientific), and adaptable fluorescence filter sets. Peak channel excitation and emission wavelength/bandpass in nm were 350/50ex and 457/50em for DAPI, 490/20ex and 528/38em for FITC, and 555/28ex and 617/73em for TRITC. Image capture and analyses were controlled using the software package NIS Elements AR 3.0 (SP 1). All three channels were captured for images of time course filters while TRITC was omitted for bacterial isolate filters. The exposure times were 2 s for DAPI, 12 s for FITC, and 10 s for TRITC for slides of the time course incubation, while they were 1 s for DAPI and 2 s for FITC for slides of the bacterial isolates. Images were collected for 10 haphazardly selected fields (area = 6022 μm^2) from each filter.

To enumerate cells from captured images, intensity thresholding levels were set manually for each channel and binary layers were constructed from these levels. 'Total cells' were quantified from the DAPI binary layer. An intersected DAPI-FITC binary layer was constructed to identify green objects that also displayed DAPI signal.

We observed that autofluorescent pigments from cyanobacteria and picophytoeukaryotes displayed high intensity in the TRITC channel and somewhat less intense signal in the FITC channel. To distinguish these cells, an intersected DAPI-FITC-TRITC binary layer was also constructed. 'EdU-labeled cells' were defined as the difference between the two intersected binary layers. Size restrictions were set such that the lower limit on cell area was always 0.02 μm^2 while the upper limit varied by image (generally 3.0 - 5.0 μm^2) to exclude protists and amorphous particles.

To quantify signal intensity in EdU-labeled isolate cells, we first subtracted image-specific background signal. Then binary layers were constructed using the DAPI channel as described above and saved. DAPI and FITC channels were then exported separately for each image file and the FITC monochannel image was overlayed with the saved DAPI binary layer. FITC channel data were collected for cells defined by this binary layer and included individual cell area (in μm^2), sum intensity (the integrated intensity for all pixels within a cell area), and mean intensity (the sum intensity within a cell area divided by the number of pixels within the area).

EdU inhibitory effect on bacterial production:

Seawater was collected from SIO Pier on October 21, 2009 at 09:00 (19.1°C; salinity= 33.5; chlorophyll = 0.63 μg/L). Seawater was dispensed into duplicate polycarbonate bottles (Nalgene) and amended with 0, 0.02, 0.1, or 1.0 μM EdU. Incubation bottles were maintained at 20°C. Subsamples were preserved at 0, 1, 2, 4,

and 7 h by adding formaldehyde to 2% final concentration and cooling to 4°C. Aliquots (5 mL) were collected onto 0.2-μm white polycarbonate filters (Millipore) for abundance estimates via DAPI staining. Additional subsamples were removed at the same timepoints to determine protein production estimates via ^{3}H-leucine incorporation (Kirchman*, et al.*, 1985, Simon & Azam, 1989) by the microcentrifugation protocol (Smith & Azam, 1992).

Time course of EdU incorporation:

Seawater was collected from SIO pier on August 19, 2009 at 11:30 (22.9°C; salinity= 33.5; chlorophyll = 0.61 μg/L). Seawater was dispensed into duplicate polycarbonate bottles (Nalgene) for the following treatments: EdU-amended (1 μM), EdU-amended containing 4% formaldehyde (killed control), and non-amended (blank). Bottles were maintained during the timecourse in a natural spectrum light incubator (20°C; Thermo) on a 12 h light/dark cycle. Subsamples were preserved at 0, 3, 5, 7, 9, and 26 h by adding formaldehyde to 2% final concentration and cooling at 4°C, except for killed controls whereby subsamples were simply cooled at 4°C. After 15-min fixation, 2 mL were filtered on 0.2-μm glass filters (Anodisc). Filters were rinsed with 2 mL PBS, dried, and stored at -20°C until processing. For control and killed control, bacteria filters (2 mL) were made at 0, 9, and 26 h only. All filters were processed with click chemistry as described above (also Fig. 1).

Comparison with 3H-TdR microradiography

Seawater was collected from SIO Pier on April 6, 2010 at 09:30 (16.5°C; salinity= 33.4; chlorophyll = 1.39 μg/L). Seawater (5 mL) was dispensed into duplicate tubes (Falcon), amended with 1.0 μM EdU or 20 nM ^{3}H-thymidine, and incubated for 1 h at 20°C. Subsamples were preserved as above and 2 mL were filtered onto Anodisc (EdU) or polycarbonate (^{3}H-TdR) membranes. EdU-labeled cells were detected and enumerated as described above. Membranes containing ^{3}H-thymidine labeled cells were processed for microradiography (using 3 d exposure to emulsion crystals) and enumerated via epifluorescence microscopy (Fuhrman & Azam, 1982). Data were acquired from 10 microscopy fields each for replicate EdU filters and one ^{3}H-TdR filter.

Isolates at different growth rates:

Tests on the relationship between growth rate and signal intensity in cultured isolates were designed following similar studies of BrdU incorporation (Hamasaki*, et al.*, 2004). Briefly, four marine isolates (Table 1) were grown in a seawater medium for 2 d. An aliquot of each culture was inoculated into duplicate tubes of 8 mL fresh seawater medium (SWM) and incubated for 2 h at room temperature. The experiment was then started by amending cultures with EdU (1 μM). Subsamples (1 mL) were collected at 0, 2, 5, and 9 h and fixed with 4% formaldehyde. Aliquots of the subsamples (0.1 mL) were collected onto Anodisc filters, rinsed with 2 mL PBS, dried, and stored at -20°C until processing. Filters for the 5 h time point were

processed to determine individual cell EdU signal intensity as described above. Total cell abundance was determined for all time points using separate filter pieces that were DAPI stained. The specific growth rate of each isolate was estimated using the abundance results for 2, 5, and 9 h. A follow-up experiment on isolate SB19 tested whether EdU incorporation was limited to growing cells. The test was the same as above but included a formaldehyde-fixed incubation and live (non-fixed) cells incubated on ice.

EdU/TdR competition:

Four marine isolates were grown in SWM for 2 d and then transferred into fresh medium for 2 h, as above. Duplicate culture aliquots were then amended with EdU only (20 nM), or co-amended with thymidine (1 μM) and EdU (20 nM). Subsamples were collected at 5 h and then fixed and processed for EdU signal as above.

Fish feces associated bacteria:

Feces were acquired from a stonefish (*Synanceia* sp.) housed at Birch Aquarium at Scripps. The sample was rinsed once with filtered seawater, broken into smaller pieces using a pipette tip, and resuspended in 5 mL filtered seawater. The slurry was then amended with EdU (1 μM) for a 1 h incubation at 22ºC before being fixed and processed as described above.

RESULTS

Method optimization for detecting EdU-labeled bacteria

Among three membrane filter types tested for collecting formaldehyde-fixed bacteria, Anodisc filters produced the lowest background for both DAPI and EdU-specific AlexaFluor-488 signals. Labeled cells could be observed on white polycarbonate filters but showed higher DAPI and FITC channel background fluorescence relative to Anodiscs. Cells on black polycarbonate filters produced relatively dim DAPI signal as well as dim AlexaFluor-488 signal in the FITC channel.

Polycarbonate and Anodisc membrane filters treated with lysozyme to lyse cells displayed high FITC channel fluorescence background with variable and inconsistent signal from AlexaFluor-488-labeled cells. In some regions of a single lysozyme-treated filter, labeled cells were abundant and bright against high background fluorescence while in other regions labeled cells were few and dim against slightly less intense background fluorescence. Tests that combined pepsin with lysozyme did not reduce the background fluorescence. Conversely, untreated filters displayed far less background fluorescence than those that were lysozyme-treated in both DAPI and FITC channels. Also, more labeled cells could be observed on untreated than on lysozyme-treated filters.

Among four methods tested for applying click-chemistry reaction cocktail, the 'slip-inversion' method using either ethanol-washed coverslips or sterile hydrophobic coverslips provided sufficient coverage of the membrane filter surface and consistent EdU-labeling among cells. Meanwhile, with regard to counterstaining and mounting

methods tested on Anodisc filters, DAPI signal intensity for cells stained by VectaShield-DAPI was relatively weak and the AlexaFluor-488 signal was relatively less intense. By contrast, staining via perfusion on a drop of DAPI solution followed by mounting in glycerol-based anti-fading solution provided intense cell DAPI signal with low background and relatively intense AlexaFluor-488 signal with low background. Cell margins were well defined for both DAPI and AlexaFluor-488 signals. DAPI and AlexaFluor-488 signal intensities on click-processed filters decreased markedly within two days of slide storage at -20°C.

Based on these results, a protocol was adopted (Fig. 1) for detection of EdU-labeled bacteria on sample membrane filters produced during labeling time course incubations of coastal seawater assemblages and those produced during labeling incubations of bacterial isolates at different growth rates, below.

EdU effect on bacterial production

Total cell abundances in whole (non-filtered) seawater cultures incubated at different EdU concentrations remained constant over 7 h (Fig. 3, top panel). Meanwhile, carbon production at different EdU concentrations generally increased over 7 h (Fig. 3, bottom panel). While rates varied at different times for any one EdU concentration, production was always positive for all EdU concentrations and did not differ from unamended controls. Meanwhile, the percentage of EdU-labeled cells (±S.D.) after 7 h incubation was 7.0 ±0.27%, 7.9 ±1.14%, and 9.4 ±2.01% for 20 nM,

100 nM, and 1 μM EdU, respectively, with no significant difference among concentrations.

Labeling time course for bacteria in coastal seawater

The abundance of EdU-labeled bacteria in EdU-amended whole (non-filtered) seawater incubations, as well as the total bacteria abundance as observed via DAPI, slightly increased over a 26 h time course (Fig. 4). The percentage of labeled cells ranged 6.2% to 17.9% (Fig. 4, right panel). EdU-labeled bacteria abundance in EdU-amended seawater was higher than those in parallel control incubations of unamended seawater and EdU-amended-formaldehyde-fixed seawater (Fig. 4). In the unamended control, the percentage of apparently labeled cells ranged 1.4% to 6.5%, while in the formaldehyde-fixed seawater control it ranged 1.9% to 4.4% (Fig. 4, right panel).

Labeling of Growing Bacterial Isolates

Specific growth rates among four isolates ranged 0.052 to 0.135 h^{-1} with doubling times ranging 7.4 to 19.2 h (Table 1). *Vibrio corallillyticus* cells displayed the highest integrated and cell area-specific EdU signal intensity, while Cytophaga strain SB19 displayed the lowest (Table 1). Specific growth rate was not correlated with EdU signal intensity among these isolates. In this regard, BBAT1 and SB19 had relatively high specific growth rates but displayed relatively low cell signal intensity (Table 1). Specific growth rate was not determined for *V. corallilyticus* because abundance estimates were made difficult by clustering and amorphous cell shape. In

an additional test for incorporation specificity, the four isolates became labeled after 5 h in 20 nM EdU, but did not become labeled in treatments that contained both EdU (20 nM) and thymidine (1 μM or 50X higher concentration than EdU). In another test, growing cells of isolate SBD8 incubated in EdU (1 μM) became labeled, but formaldehyde-fixed cells or live cells incubated on ice at the same EdU concentration were not.

Comparison of EdU with ^{3}H-thymidine microautoradiography

In a 1 h incubation of coastal seawater, percent labeled cells was 22.8% and 14.4% for replicate filters processed via the EdU method and 17.0% for one filter processed with ^{3}H-TdR-microautoradiography. In formaldehyde-fixed parallel incubations, the apparent percent labeled cells was 8.1 and 6.8% for replicate EdU filters while there was no apparent labeling via ^{3}H-TdR microautoradiography.

Cellular localization of EdU signal

Cellular localization of the EdU signal varied among four bacterial isolates growing in enriched seawater media. Distinct spots and patches were visible within individual cells of *V. corallillyticus* and *Flexibacter* SBD8 (Fig. 5B and 5C), while EdU signal within BBAT1 cells was distributed more homogenously. Among bacteria in natural coastal seawater, some rod-shaped cells observed in the DAPI channel displayed high EdU signal at each pole with sparse signal in the cell mid-section (Fig. 5A). For these cells, a septum was not visible in the DAPI channel. In general,

locations within growing cells that displayed diminished signal in the DAPI channel displayed highly intense EdU signal in the FITC channel. This was observed for both cultured isolates and natural assemblage cells. Likewise, some cocci- and rod-shaped cells displaying relatively dim DAPI signal homogenously throughout the cell displayed extremely intense FITC signal over the same cell area.

DISCUSSION

The detection of DNA synthesis via EdU incorporation and click chemistry provides a new approach for observing growth among marine bacteria. The approach complements other methods designed to quantify individual cell growth and metabolism, such as ^{3}H-TdR microautoradiography, and may be useful for addressing long-standing questions on growth physiology, e.g. what portion of bacteria in the ocean are dormant? We discuss methodological considerations and consider the feasibility of using EdU to quantify cell growth rates and observe DNA synthesis within individual cells in the environment.

EdU detection, potential inhibition, and specificity

Following the filtration of seawater samples or fish feces samples, processing for the visualization of EdU-labeled cells could be completed within 1 h (Fig. 1). Importantly, we found that cell lysis was not necessary to detect labeled cells and that lysozyme treatment was in fact detrimental because it caused high fluorescence background on filters. A click reaction incubation time of 30 min was used for all

tests in this study but it may be possible to shorten the incubation without compromising individual cell signal intensity or percentage of EdU-labeled cells. The method permits detection of labeled cells on membrane filters, which preserves microscale cell distribution better than centrifugation- or filter-transfer-based methods like those that have been used in microautoradiography and BrdU immunochemistry. Labeled cells could be observed 18 months after filtration onto polycarbonate membranes that were subsequently stored dry and unprocessed at -20°C. Thus the method is amenable to field studies where time available for sample processing may be limited.

To test whether a separate DAPI-staining step could be eliminated, we tried amending the EdU cocktail directly with DAPI. We observed that bacteria became stained with DAPI but caused high background fluorescence on Anodisc filters in both DAPI and FITC channels, thus decreasing signal to background ratio for EdU-labeled cells. This may have been caused by nonspecific binding of DAPI to the filter and/or nonspecific interactions of the AlexaFluor-azide conjugate with adherent DAPI. Additionally, we observed that cells processed via click reaction displayed diminished DAPI intensity and cyanobacteria displayed diminished autofluorescence. Parallel incubations of reaction cocktail with or without Cu(II) demonstrated that this component alone accounted for diminished signal intensity, though it is not clear whether signal loss was due to Cu(I)-catalyzed click cycloaddition or direct interactions between ionic Cu and cellular macromolecules.

Inhibitory effects of EdU on bacteria at concentrations ranging from 20 nM to 1 μM were minimal, as demonstrated by increased productivity in coastal seawater bacterial assemblages over 7 h incubation (Fig. 3). These observations suggest that incubations up to 7 h at concentrations up to 1 μM EdU will permit cell labeling while inducing minimal perturbation to natural bacterial assemblages. In preliminary tests we observed 10% to 30% lower production and cell abundances after 24 h in EdU-amended treatments relative to unamended controls, suggesting that some inhibition may occur after 24 h incubation. Use of the method for detecting percentages of growing bacteria should therefore be limited to no greater than 7 h incubation in EdU.

We considered the specificity of the click reaction in detecting EdU. A small percentage of apparently labeled cells were observed at the start of the time course experiment in EdU-amended treatments, in unamended controls, and in formaldehyde-fixed controls (Fig. 4). Several possibilities may account for these observations. First, it may be that some nonphotosynthetic, presumably heterotrophic bacteria emitted green autofluorescence. Indeed, *Synechoccoccus* and other picophytoplankton contained autofluorescent pigment emissions that were detected in the FITC channel, and we subtracted these during our quantification of labeled cells (see Methods). However in DAPI stained filters that did not undergo click reaction, we observed very few apparent green autofluorescent bacteria (though we did see green autofluorescent protists and amorphous particles). A second possibility is that wide fluorescence emission spectra from DAPI bled into the FITC channel. This is inconsistent, though, with our observation that most DAPI stained cells displayed no colocalized signal

above background in the FITC channel. A third possibility is that the AlexaFluor-azide conjugate underwent click reaction with naturally occurring alkynes in marine bacteria. Indeed, alkyne-containing enediynes have been found in *Salinospora* sp., a bacterial genus that is commonly cultured from marine sediments (Udwary*, et al.*, 2007). However, it is not known whether enediynes exist in pelagic bacteria. Future studies will need to elucidate the mechanism(s) that contribute to background EdU signal via click chemistry in seawater. Such studies may be aided by the use of azides conjugated to other fluorophore colors (e.g. Pacific Blue; Invitrogen), which may help differentiate potential interference effects from green and red autofluorescent pigments.

EdU incorporation and bacterial growth

Despite apparent nonspecific background signals, the percentage of labeled cells in natural seawater assemblages increased during the time course experiment (Fig. 3), growing marine isolates became labeled (Table 1 and Fig. 5) while nongrowing SB19 isolate cells that were fixed or incubated live on ice did not, and specific signal intensities varied among cells in cultures (Table 1). Together these results suggest that the method detects growing bacteria and that growing cells incorporate different quantities of EdU.

Given the ecological implications, it is imperative to estimate the range of growth rates that the method can detect. For three isolates that incorporated EdU, specific growth rates ranged 0.052 to 0.135 h^{-1} (19.2 to 7.4 h doubling time). If we

assume that signal intensity of these cells is applicable to natural assemblages of bacteria, then labeled cells with equal or greater cell-specific fluorescence intensity observed in the natural seawater incubations were growing at or faster than 0.052 h^{-1} (19.2 h doubling time). The community-average specific growth rate in filtered seawater cultures was 0.076 h^{-1} (between 4 and 8 h incubation; Fig. 3) and in whole seawater was 0.013 h^{-1} (between 3 and 9 h incubation; Fig. 4). These community-average rates presumably reflect fast- and slow-growing cells, and slow-growing cells may be proportionately more abundant in assemblages with lower community-wide rates. We did not calculate the lowest observable growth rate because the detection limit was not determined, though future efforts to determine it should compare cell specific ^{3}H-EdU incorporation with fluorescence signal intensity.

Quantification of cell-specific growth rates using EdU would require comparison of EdU signal against well-calibrated standard curves. In a previous study of BrdU-labeled isolates, Hamasaki and colleagues (2004) found a positive linear correlation between cell specific signal intensities and growth rates. In the current study, neither integrated nor cell-area specific EdU signal intensity correlated with specific growth rate among the three bacterial isolates for which growth rate was determined (Table 1). Both experimental conditions and taxa specificity may have contributed to this apparent incongruity. However, growth rates for the three isolates (0.052 to 0.135 h^{-1}) were all higher than those reported in the BrdU study (0.015 to 0.037 h^{-1}). Also, the isolates tested in the present study were more taxonomically diverse than the BrdU study, which tested five *Cytophaga-Flavobacter-Bacteroides*

strains. Future efforts toward the goal of accurately determining individual cell growth rates in environmental bacteria may need to consider taxon-specific EdU incorporation efficiency.

The percentage range of cells that were labeled over 7 h during the time course experiment (6.2% to 17.9%) overlapped with previous estimates of ^{3}H-TdR incorporation in free-living marine bacteria (Fuhrman & Azam, 1982, Douglas*, et al.*, 1987, Pedros-Alio & Newell, 1989). This is consistent with frequent observations that a large fraction of bacteria in natural assemblages are non-growing or growing at very slow rates as estimated via bulk ^{3}H-TdR incorporation.

Cellular localization of EdU

The observed intracellular EdU signal distribution in cultured isolates and natural assemblages (Fig. 5) likely indicates replicating cells within these populations. Mid-cell septa are one observable characteristic of replicating bacteria and the quantification of septated cells (i.e. frequency of dividing cells or FDC) has been used to estimate assemblage growth rates (Hagstrom*, et al.*, 1979, Riemann*, et al.*, 1984). While prior estimates of FDC were based on DAPI- or acridine orange (AO)- staining, EdU labeling may provide an alternate method.

In our study, some non-septated cells displaying DAPI signal throughout contained EdU signal localized to two or more regions within the DAPI area. This suggests that 'old' DNA was distributed throughout these cells while nascent DNA was specifically localized. Such cells would not be counted as 'dividing' using DAPI

or AO but they would be counted if FDC quantification were based on EdU-labeling. Interestingly, not all DAPI-based septated cells displayed EdU-label. This is surprising since all daughter cells produced during incubation should contain newly synthesized DNA with incorporated EdU. However, labeling would not occur if the cells were not capable of EdU uptake, or if they did not replicate during the time course because they were growing too slowly, or if they went into 'stasis' prior to the start of the labeling incubation.

For *Flexibacter* SDB8, many non-septated cells displayed EdU signal at the far poles (Fig. 5Cii), which may reflect poleward partitioning of newly synthesized DNA near the origin of replication (*oriC*) during replication, such as has been described in *E. coli* (Draper & Gober, 2002). We cannot be certain that these patterns reflect intracellular distribution of nascent DNA. If they do, then EdU incorporation provides an advantage over ^{3}H-TdR microautoradiography in that quantification of silver crystal cluster size via the latter method does not provide sufficient resolution to observe intracellular distribution of ^{3}H-TdR-labeled DNA, even though it does permit relative comparisons of substrate incorporation (Cottrell & Kirchman, 2003). Furthermore, EdU labeling could provide a more time-efficient method to BrdU labeling for studies of chromosomal segregation in model organisms such as *B. subtilis* (Lewis & Errington, 1997). With regard to signal amplification of EdU label, Salic and Mitchison (2008) found that the total intensity of EdU signal can be increased in eukaryotic cells through repeated incubation with fresh reaction mixture

without a change in the signaling distribution. Such an approach applied to labeled marine bacteria may improve the detection of low-concentration EdU loci within cells.

Summary

The use of EdU incorporation and click chemistry to detect growing marine bacteria offers a complementary measurement to the detection of ^{3}H-TdR via microautoradiography or BrdU via immunochemistry. The EdU method is much simpler and requires considerably less time to observe growing cells. In this study, bacterial assemblages in coastal California seawater and in fish feces were tested (Fig. 2). However, I recognize that in order for the method to be broadly accepted, it will need to be tested in oligotrophic seawater, brackish waters, and other representative environments. Future studies that quantify detection limits and EdU:TdR incorporation ratios, among other factors, should enable the conversion of EdU signal into assemblage and/or individual cell production rates. Furthermore, EdU incorporation combined with fluorescence in situ hybridization should help determine species-specific *in situ* growth rates at the individual cell level. Adaptation of this approach may improve individual-cell-based microbial ecology and contribute to our understanding of the wide spectrum of microscale interactions that sustain biogeochemical processes in marine ecosystems.

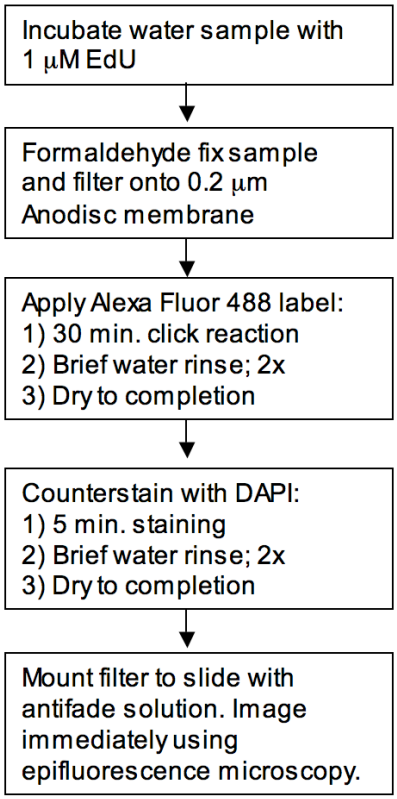


Figure 6-1: Overview of protocol for ethynyldeoxyuridine incorporation into marine bacteria and detection using click chemistry.

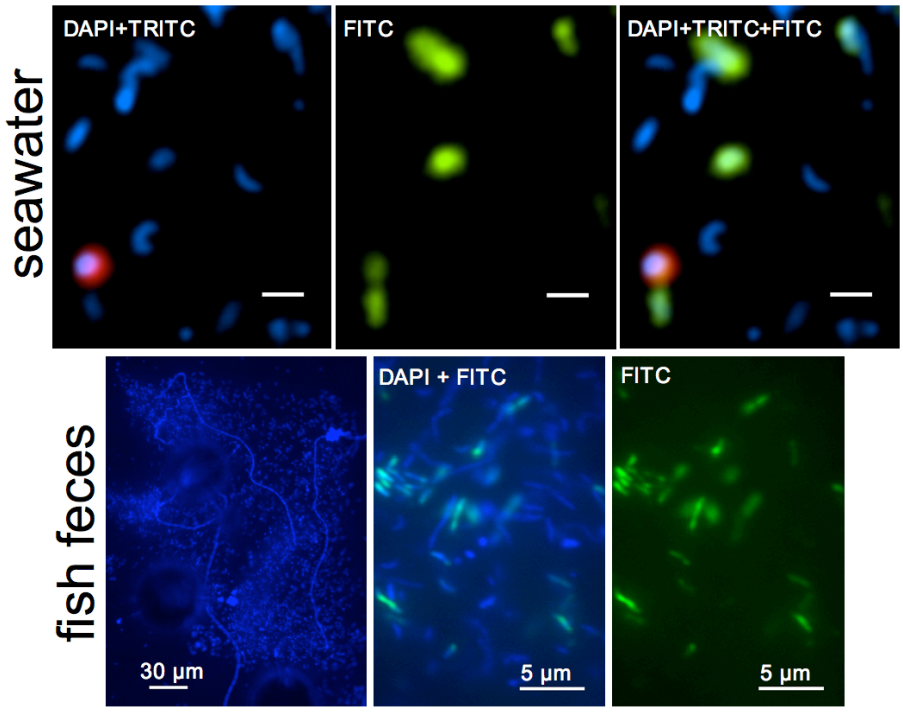


Figure 6-2: Epifluorescence microscopy images of ethynyldeoxyuridine (EdU)-labeled bacteria from coastal seawater (top row) and stonefish feces (bottom row). For seawater, total bacteria including one autofluorescent *Synechococcus* cell (red) are shown in the DAPI and TRITC channels (left), while EdU-labeled cells (green) are shown in the FITC channel (middle) and in the combined-channel image (right). Scale bar = 1 μm. For fish feces, a DAPI-stained fecal aggregate (left panel) is highly colonized with bacteria. Higher magnification images (middle and right) show large cells, many of which are labeled with EdU (green). Both seawater and fish feces samples were incubated with EdU, then click chemistry was used to tag incorporated EdU molecules with AlexaFluor 488 azide, which is excitable in the FITC channel.

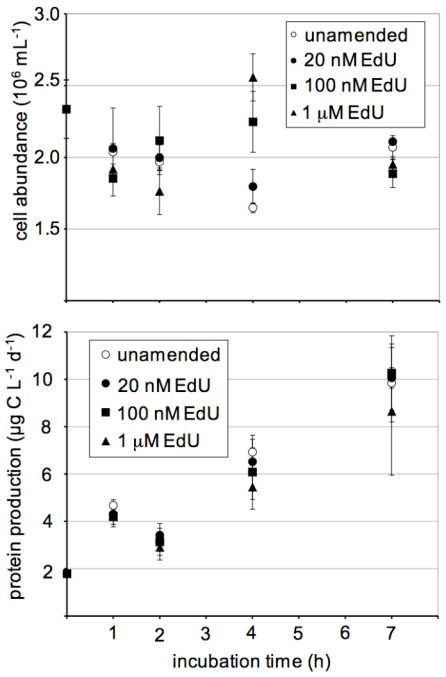
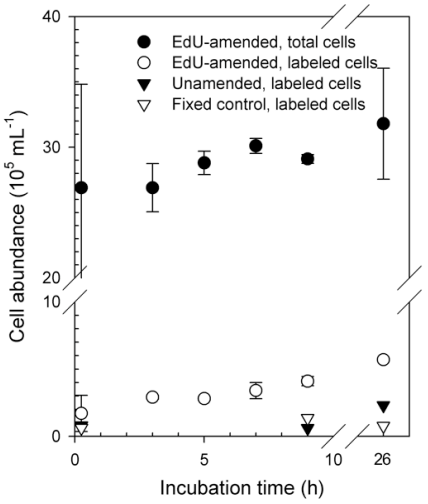


Figure 6-3: Time course showing bacterial cell abundance (top) and protein production (bottom) in coastal seawater at four concentrations of ethynyldeoxyuridine. Error bars show ±S.E. of replicates**.**



Incubation time (h)	% labeled cells (mean) EdU-amended	Unamended control	EdU-amended Fixed control
0.25	1.7, 8.7 (6.2)	2.5	1.9
3	10.5, 10.9 (10.7)	n.d.	n.d.
5	8.9, 10.3 (9.6)	n.d.	n.d.
7	9.1, 13.5 (11.2)	n.d.	n.d.
9	15.0, 13.0 (14.0)	1.4	4.4
26	21.6, 15.2 (17.9)	6.5	3.8

Figure 6-4: Time course showing total cell abundance (closed circles) and EdU-labeled cell abundance (open circles) in EdU-amended coastal seawater. Replicate unfiltered seawater samples were amended at 0 h with 1 μM ethynyldeoxyuridine. Abundances for unamended seawater controls (closed triangles) and EdU-amended formaldehyde-fixed controls (open triangles) are also shown. Error bars show ±S.E. The percentage of EdU-labeled and apparently-labeled cells (right) correspond with abundance data presented in the graph (left). Percentage values for replicate filters (with the mean in parentheses) are shown, and control values for a single filter (shown in right two columns) were calculated from total abundances in corresponding treatments (data not shown in graph).

Table 6-1: Specific growth rates and individual cell EdU signal intensity for four marine bacterial isolates. Total cells (*n*) were DAPI-stained cells and 'labeled cells' were any that displayed EdU signal above background. Growth rates were determined from DAPI-based count; mean values for duplicate trials are shown. Mean intensity values are reported for *n* cells per isolate. Integrated cell signal intensity was divided by pixel area to give cell area-specific signal intensity. [a]Coefficient of variance (C.V.)

	n	% labeled cells	Specific growth rate (h^{-1})	Doubling time (h)	Integrated EdU signal intensity total rfu $cell^{-1}$ (±S.E.)(C.V.)[a]	Cell area-specific EdU signal intensity rfu $pixel^{-1}$ (±S.E.)(C.V.)[a]
Vibrio corallilyticus	239	99.2	n/d	n/d	2.25×10^5 ($\pm 1.98 \times 10^4$)(1.35)	311 (±23.5)(1.16)
Flexibacter sp. SBD8	420	100	0.052	19.2	0.27×10^5 ($\pm 0.13 \times 10^4$)(0.99)	105 (±4.1)(0.80)
Roseobacter sp. BBAT1	261	100	0.112	8.9	0.08×10^5 ($\pm 0.03 \times 10^4$)(0.75)	32 (±1.20)(0.57)
Cytophaga strain SB19	304	96.4	0.135	7.4	0.04×10^5 ($\pm 0.04 \times 10^4$)(2.02)	12 (±0.90)(1.24)

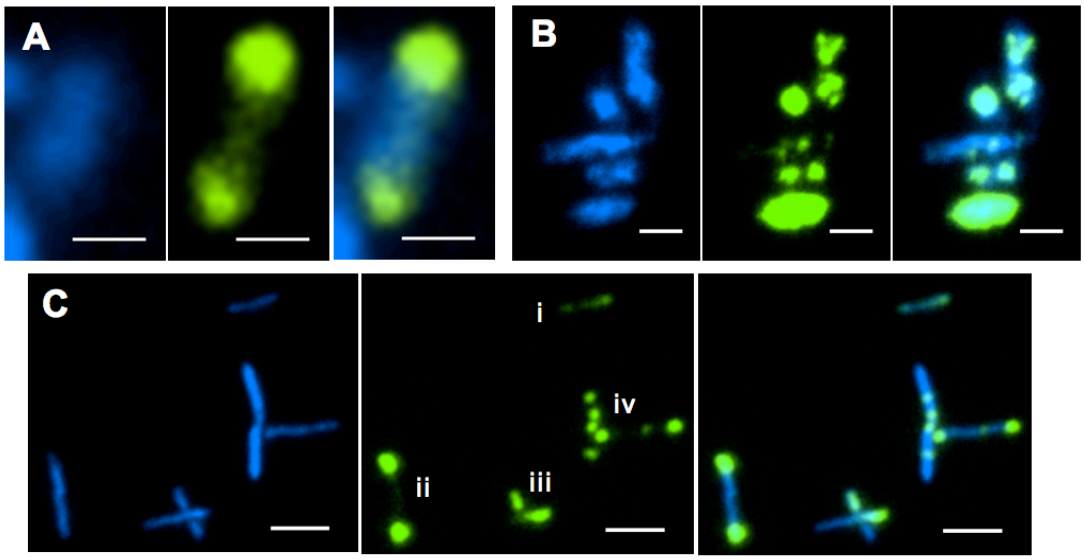


Figure 6-5: Localization of signal in EdU-labeled bacteria. Each set of images shows DAPI signal (left panel), EdU signal (middle), and combined signals (right). (A) Representative bacterium from mixed natural assemblage showing DAPI signal mid-cell and EdU signals at each pole. Scale bar = 0.5 μm. (B) A cluster of five EdU-labeled *Vibrio coralliilyticus* cultured cells showing both patchy and homogenous EdU localization. Scale bar = 2 μm. (C) Cultured cells of *Flexibacter* sp. SBD8 displaying several distinct patterns of EdU localization including (i) relatively even distribution throughout cell, (ii) at both poles, (iii) at one pole, and (iv) at multiple mid-cell sites. Scale bar = 2 μm.

Acknowledgements

We thank J. Nguyen for laboratory assistance and F. Malfatti, M. Garren, B. Pedler, and K. Rypien for helpful discussions. Support was provided byNational Science Foundation grant (OCE0648116); the Global Environment Facility/Coral Reef Targeted Research program; a contract from Gordon and Betty Moore Foundation to F. Azam; and an NSF Graduate Student Research Fellowship to S. Smriga.

Chapter 6 is currently being prepared for submission for publication: Smriga S, Samo T, and Azam F (in prep) Use of ethynyldeoxyuridine and click chemistry to detect DNA synthesis in marine bacteria. The dissertation author was the primary investigator and principal author of the manuscript.

REFERENCES

Azam F & Malfatti F (2007) Microbial structuring of marine ecosystems. *Nature Reviews Microbiology* **5**: 782-791.

Bidle KD & Azam F (2001) Bacterial control of silicon regeneration from diatom detritus: Significance of bacterial ectohydrolases and species identity. *Limnology and Oceanography* **46**: 1606-1623.

Cottrell MT & Kirchman DL (2003) Contribution of major bacterial groups to bacterial biomass production (thymidine and leucine incorporation) in the Delaware estuary. *Limnology and Oceanography* **48**: 168-178.

del Giorgio PA & Gasol JM (2008) Physiological structure and single-cell activity in marine bacterioplankton. *Microbial Ecology of the Oceans,*(Kirchman DL, ed.), pp. 243-298. Wiley-Blackwell.

Douglas DJ, Novitsky JA & Fournier RO (1987) Microautoradiography-based enumeration of bacteria with estimates of thymidine-specific growth and production rates. *Marine Ecology-Progress Series* **36**: 91-99.

Draper GC & Gober JW (2002) Bacterial chromosome segregation. *Annual Review of Microbiology* **56**: 567-597.

Fuhrman JA & Azam F (1982) Thymidine Incorporation as a Measure of Heterotrophic Bacterioplankton Production in Marine Surface Waters - Evaluation and Field Results. *Marine Biology* **66**: 109-120.

Hagstrom A, Larsson U, Horstedt P & Normark S (1979) Frequency of dividing cells, a new approach to the determination of bacterial-growth rates in aquatic environments. *Applied and Environmental Microbiology* **37**: 805-812.

Hamasaki K, Long RA & Azam F (2004) Individual cell growth rates of marine bacteria, measured by bromodeoxyuridine incorporation. *Aquatic Microbial Ecology* **35**: 217-227.

Kirchman D, Knees E & Hodson R (1985) Leucine Incorporation and Its Potential as a Measure of Protein-Synthesis by Bacteria in Natural Aquatic Systems. *Applied and Environmental Microbiology* **49**: 599-607.

Kolb HC, Finn MG & Sharpless KB (2001) Click chemistry: Diverse chemical function from a few good reactions. *Angewandte Chemie-International Edition* **40**: 2004-2021.

Lewis PJ & Errington J (1997) Direct evidence for active segregation of oriC regions of the Bacillus subtilis chromosome and co-localization with the SpoOJ partitioning protein. *Molecular Microbiology* **25**: 945-954.

Noble RT & Fuhrman JA (1998) Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquatic Microbial Ecology* **14**: 113-118.

Pedros-Alio C & Newell SY (1989) Microautoradiographic study of thymidine uptake in brackish waters around Sapelo Island, Georgia, USA. *Marine Ecology-Progress Series* **55**: 83-94.

Pernthaler A, Pernthaler J, Schattenhofer M & Amann R (2002) Identification of DNA-synthesizing bacterial cells in coastal North Sea plankton. *Applied and Environmental Microbiology* **68**: 5728-5736.

Riemann B, Nielsen P, Jeppesen M, Marcussen B & Fuhrman JA (1984) Diel changes in bacterial biomass and growth-rates in coastal environments, determined by means of thymidine incorporation into DNA, frequency of dividing cells (FDC), and microautoradiography. *Marine Ecology-Progress Series* **17**: 227-235.

Salic A & Mitchison TJ (2008) A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 2415-2420.

Simon M & Azam F (1989) Protein-Content and Protein-Synthesis Rates of Planktonic Marine-Bacteria. *Marine Ecology-Progress Series* **51**: 201-213.

Smith DC & Azam F (1992) A simple, economical method for measuring bacterial protein synthesis rates in seawater using 3H-leucine. *Marine Microbial Food Webs* **6**: 107-114.

Smith DC, Simon M, Alldredge AL & Azam F (1992) Intense Hydrolytic Enzyme-Activity on Marine Aggregates and Implications for Rapid Particle Dissolution. *Nature* **359**: 139-142.

Udwary DW, Zeigler L, Asolkar RN*, et al.* (2007) Genome sequencing reveals complex secondary metabolome in the marine actinomycete Salinispora tropica. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 10376-10381.

CHAPTER 7: Conclusions

Coral reef fish feces are understudied class of marine organic particles that represent an integration of ingested prey. Some consistent themes emerge from the studies presented in this thesis. First, fish feces host high bacteria abundances and movement of fecal particles can transport bacteria within coral reefs. Second, some bacterial species (phylotypes) associated with fish feces can co-occur in corals, seawater, and organic particles. Third, bacterial community composition in feces may be dominated by Vibrionaceae in many fish species. However, there are distinct taxa that may be feces-specific and distributed among diverse fish species. Fourth, some bacteria can persist in egested feces and this may act as a strategy for long-term survival in the environment. Fifth, fecal bacteria can experience rapid growth and a newly adapted technique should enable us to determine individual cell growth rates for bacteria associated with feces-coral interactions. Finally, corals and coral-associated bacteria may be adapted to acute smothering by parrotfish feces.

A primary focus of this thesis was ultimately on community species composition and dynamics as determined via 16S rRNA genes. A central question that was only tangentially addressed is: what are the bacteria doing? Certainly they were able to grow, and increases in cell abundance were very likely supported by dissolved carbon and nutrients available in the feces. The bacteria may have also been expressing hydrolytic enzymes that degraded the fecal organic matrix, which includes proteins and polymers of ingested microalgae. While the results could not disassociate bacterial enzyme activity from eukaryotic activity, the high bulk rates are consistent with enzyme activity estimates for marine pelagic particles, where coupling between

enzymatic hydrolysis and incorporation supports bacterial growth. In parrotfish feces, bacterial enzymes probably degrade algal fragments to create DOM, and bacteria incorporate it into biomass during growth. If this conversion process really occurs, it would be consistent with the 'microbial loop' model for biological cycling in the ocean (Pomeroy 1974; Azam et al. 1983). An important question to address in the future is whether protists graze upon bacteria in egested feces, and whether the biomass incorporated via bacterivory is transferred into higher trophic levels.

The parrotfish feces organic milieu likely includes partially digested algal fragments, detached fish epithelial cells, bile acids, and chitinous fragments. This organic matter is intertwined within the inorganic carbonaceous fragments that make up the bulk physical structure of a fecal pellet, and bacterial interactions may shape the microscale architecture of the organic matter. Do the different types of OM become colonized by different bacterial species? Are single bacterial phylotypes adapted to utilize diverse organic substrates? Are there microenvironments in which robust bacterial growth occurs while others where bacteria merely survive? While the results presented in the preceding chapters cannot resolve answers to these questions, they provide a first attempt to attain insights at the bulk scale.

Interest in gut and feces microbiology is expanding as exemplified by numerous studies recently published on these topics. Most of them focus on human and terrestrial animals, but already the field is broadening to address freshwater and marine fish as well as other aquatic organisms. The findings undoubtedly hold promise for exciting discoveries that enhance our basic understanding of biological

processes. They may also help inform policy decisions regarding fish conservation, aquaculture, and other issues that lie at the nexus between ocean and human health.

Molecular approaches, such as those described in this thesis, continue to revolutionize our understanding of the in situ functioning of microbes within the coral holobiont. Moreover, these discoveries may influence broader unifying concepts in coral reef conservation and environmental biology.

REFERENCES

Azam F, Fenchel T, Field JG, Gray JS, Meyer-Reil LA & Thingstad F (1983) The Ecological Role of Water-Column Microbes in the Sea. *Marine Ecology-Progress Series* **10**: 257-263.

Pomeroy LR (1974) Oceans food web, a changing paradigm. *Bioscience* **24**: 499-504.