



Response of the microbial community to coral spawning in lagoon and reef flat environments of Hawaii, USA

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ABSTRACT: The response of the microbial community to coral spawning was investigated over a period of 18 mo, from January 2006 to July 2007, in reef flat and lagoon environments of a subtropical embayment (Kaneohe Bay, Oahu, Hawaii, USA). The composition of the bacterioplankton community was characterized using terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial small-subunit (SSU) ribosomal RNA genes in parallel with measurements of microbial cell abundances, bacterial production via ³H-leucine incorporation, and seawater biochemical parameters. We observed a variable bacterioplankton community structure and 2- to 3-fold changes in the cellular abundance of microorganisms, concentrations of chlorophyll *a*, and rates of bacterial carbon production at both sites during non-spawning conditions. While shifts in the structure of the bacterioplankton community were evident for both environments following coral spawning, microbial abundances and rates of bacterial production remained largely unchanged from pre-spawning levels. Thus, it appeared that only a small fraction of the microbial community responded to the presence of coral-produced organic matter. Differences in the composition of the bacterioplankton community, cellular abundances of microorganisms, and rates of bacterial production were evident between the lagoon and reef flat sites during non-spawning conditions, probably signifying the importance of the surface flow regime for coastal reef microbial communities. Our observations indicate that the Kaneohe Bay microbial community may be more significantly affected by physical mixing processes than by organic matter loading from coral spawning.

KEY WORDS: Coral · Spawning · Marine bacteria · Microorganisms · Bacterial production · Kaneohe Bay · SSU rRNA gene · T-RFLP

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INTRODUCTION

The microbial ecology of coral reefs is increasingly recognized as an important factor to consider when investigating the health of globally threatened coral reef ecosystems. Recent research has focused on characterizing coral-associated bacterial and archaeal communities with the goals of assessing community diversity, species-specificity and the potential roles played by the microorganisms in the health of corals

(Rohwer et al. 2001, 2002, Casas et al. 2004, Kellogg 2004, Wegley et al. 2004, 2007, Beleneva et al. 2005, Bourne 2005, Bourne & Munn 2005, Guppy & Bythell 2006, Sekar et al. 2006, Lesser et al. 2007). However, planktonic marine microorganisms may also significantly affect the health of corals and reef ecosystems, though their characterization has received considerably less attention (Dinsdale et al. 2008, Garren et al. 2008). For example, blooms of potentially pathogenic microorganisms that are not typically dominant mem-

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bers of the microbial community have emerged following nutrient loading in subtropical coastal areas (Piccini et al. 2006). Understanding the response of the bacterioplankton community to nutrient and organic matter loading and other controlling factors is undoubtedly important for identifying potential health impacts for corals and other reef organisms.

Tropical and subtropical coral reef waters are oligotrophic by nature and typically harbor dissolved inorganic nitrogen and phosphorus concentrations of <0.5 and <0.1 μM , respectively (Furnas et al. 2005, Cox et al. 2006). Primary production in reef environments is generally considered to be limited by these nutrients, and phytoplankton blooms resulting from nutrient input into coastal reef systems have been documented (Furnas et al. 2005, Ringuet & Mackenzie 2005, Cox et al. 2006). Productivity in reef environments is also affected by nutrient exchange with the underlying sediment and benthic organisms, including reef-building corals (Ducklow 1990). Scleractinian corals rely on endosymbiotic dinoflagellates to fix inorganic carbon, and this carbon source not only meets the metabolic needs of corals but also makes corals a significant source of particulate and dissolved organic carbon (Ducklow 1990). Coral-produced organic matter is tightly cycled throughout the pelagic and benthic microbial food web (Crossland 1987, Ferrier-Pagès et al. 2000, Wild et al. 2004a, 2008), and is theorized to be the primary source of dissolved organic carbon supporting heterotrophic microorganisms in reef environments (Ducklow 1990, Gast et al. 1998, van Duyl & Gast 2001). Heterotrophic microbial community dynamics—and especially descriptions of the community members utilizing coral-produced organic matter—are not well resolved, but may provide a significant perspective on the microbial functioning of coral reef ecosystems.

Coral spawning events are predictable recruitment events that provide an opportunity to examine how reef microorganisms respond to large-scale additions of coral-produced organic matter. The spawned gametes are initially particulate, but unfertilized gametes rapidly degrade and dissolve, forming slicks that are sometimes visible from aerial views (Oliver & Willis 1987). Coral spawning on the Great Barrier Reef has recently been shown to affect both benthic and pelagic microbial communities (Wild et al. 2004b, Eyre et al. 2008, Glud et al. 2008, Patten et al. 2008). However, the effect of coral spawning on the composition of the microbial community has not yet been examined, and may provide significant insight into what portion of the reef microorganisms utilize coral-produced organic matter.

In the present study, we aimed to explore the response of the pelagic microbial community to additions of organic matter from coral spawning events

in Kaneohe Bay, a subtropical embayment located on the windward coast of Oahu, Hawaii, USA (Fig. 1). Kaneohe Bay is an ideal location for this study because it harbors an abundance of coral with known spawning periods (Hodgson 1985), and a number of studies have examined various aspects of microbial functioning in this environment (Landry et al. 1984, Ringuet & Mackenzie 2005, Cox et al. 2006, Hoover et al. 2006). We surveyed reef flat and lagoon sites in Kaneohe Bay over 18 mo in order to understand the factors driving the structure of the bacterioplankton community, microbial abundances and rates of bacterial production during non-spawning conditions. Based on our results from this survey and higher-resolution samplings conducted during periods of coral spawning, we conclude that the sites exhibited differential controlling processes, and that the microbial response from coral spawning at both sites was restricted to a small component of the microbial community.

MATERIALS AND METHODS

Summary of sampling. Seawater was sampled from a depth of 1 m at 2 sites (reef flat and lagoon) separated by ~600 m near Coconut Island in southern Kaneohe Bay, Oahu, Hawaii, USA (Fig. 1). The lagoon site was sampled mid-way into the lagoon and, at both sites, samples were taken directly above coral using a 1.7 l Teflon-lined Niskin bottle (General Oceanics). Samples were taken twice monthly from January 2006 to July 2007, and every 1 to 3 d during the summers of 2006 (June 10 to August 28) and 2007 (daily from June 13 to June 27 and July 10 to July 20). Sampling was conducted between 07:00 and 08:30 h. On 2 occasions, diurnal samplings were conducted at the lagoon site every 2 to 3 h: (1) following a *Montipora capitata* spawning from July 14 to July 16, 2007; and (2) under non-spawning conditions from October 8 to October 9, 2007. *In situ* measurements of temperature, salinity and pH were conducted at 1 m depth using a YSI 6600 multiparameter water quality monitoring sonde (YSI). Kaneohe Bay tidal height data were obtained from the National Oceanic and Atmospheric Association.

Nutrient analyses. Water samples for dissolved inorganic nutrients and total organic carbon (TOC) were subsampled into 150 ml polypropylene acid-washed bottles and frozen at -20° or -80°C , respectively, prior to analysis. Measurements of dissolved inorganic nutrient concentrations (NH_4^+ , $\text{NO}_3^- + \text{NO}_2^-$, NO_2^- , PO_4^{3-} , and silicate) were measured using a continuous segmented flow system consisting of a Technicon AutoAnalyzer II (SEAL Analytical) and an Alpkem RFA 300 Rapid Flow Analyzer (Alpkem). NH_4^+ was measured using the indophenol blue method (US Envi-

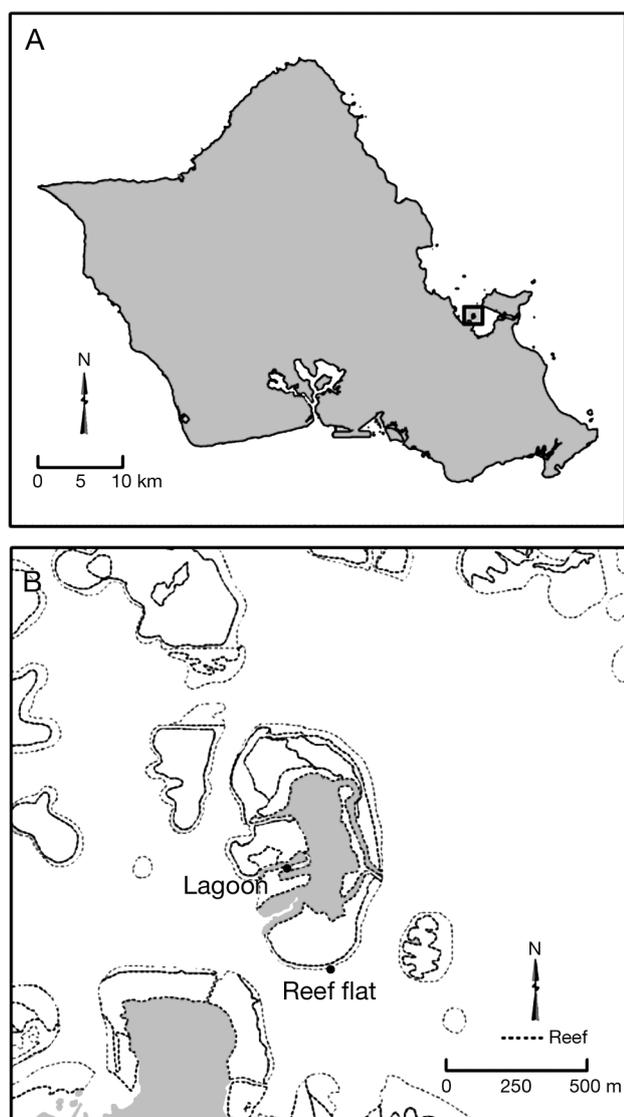


Fig. 1. Sampling sites in Kaneohe Bay, Hawaii, USA. (A) The box indicates southern Kaneohe Bay in relation to the island of Oahu, Hawaii. (B) Positions of reef flat and lagoon sampling sites surrounding Coconut Island in southern Kaneohe Bay. Dashed lines in (B) indicate living coral reefs

ronmental Protection Agency 1983), PO_4^{3-} was measured using a modified molybdenum blue method (Bernhardt & Wilhelms 1967), and $\text{NO}_3^- + \text{NO}_2^-$, NO_2^- , and silicic acid analyses were based on standard methods (Armstrong et al. 1967). Samples for TOC were acidified and oxygen purged to remove inorganic carbon, and measured using high-temperature catalytic oxidation on a Shimadzu TOC-VCSH total organic carbon analyzer (Shimadzu Scientific Instruments) (Carlson et al. 2004, Dickson et al. 2007).

Direct cell counts. Seawater samples (1 ml) for flow cytometry were fixed in a final concentration of 1% (v:v) paraformaldehyde and stored at -80°C until

analysis. Prior to analysis, the samples were stained with Hoechst 33342 (final conc. $1 \mu\text{g ml}^{-1}$) for 1 h at room temperature. Picoeukaryotic phytoplankton (hereafter picoeukaryotes), *Prochlorococcus* spp. (hereafter *Prochlorococcus*), *Synechococcus* spp. (hereafter *Synechococcus*), and non-pigmented, putatively heterotrophic bacterioplankton (hereafter heterotrophic bacteria) were enumerated using an EPICS ALTRA flow cytometer (Beckman Coulter). Discrete populations were distinguished on the basis of chlorophyll (red fluorescence, 680 nm), phycoerythrin (orange fluorescence, 575 nm), DNA (blue fluorescence, 450 nm), forward scatter, and 90° side-scatter signatures. Data analysis followed the method of Monger & Landry (1993).

Bacterial carbon production rates. The incorporation of [^3H]leucine into microbial protein was used to estimate rates of microbial production (Kirchman et al. 1985). The assays followed a protocol developed for microcentrifuge tubes (Smith & Azam 1992). Briefly, triplicate 1.5 ml seawater samples were inoculated with 20 nmol of [3,4,5- ^3H]leucine (specific activities of [3,4,5- ^3H]leucine stock were 100 to 124 Ci mmol^{-1} ; Sigma-Aldrich). A separate control sample was killed by the addition of 100 μl of 100% trichloroacetic acid (TCA) prior to isotope addition. Centrifuge tubes were placed in a turbulent water bath set at the *in situ* temperature and covered with aluminum foil. After 1 h, 100 μl of ice-cold 100% TCA was added to samples. After chilling on ice for 15 min, samples were centrifuged at $12\,000 \times g$ for 15 min to pellet the cells. Ice-cold 5% TCA (1 ml) was added to the pellet, and samples were centrifuged for an additional 5 min. Subsequently, 1 ml of ice-cold 80% ethanol was added, and the samples were again centrifuged for 5 min. The resulting cell pellets were dried overnight, resuspended in 1 ml of Univer Sol ES liquid scintillation fluid (MP Biomedicals), and counted using a Beckman LS 3801 scintillation counter (Beckman Coulter). Bacterial carbon production (BCP) rates were calculated using the conversion factor of $1.5 \text{ kg C mol}^{-1}$ leucine (Simon & Azam 1989).

Coral egg, zooplankton, and phytoplankton abundance and biomass. Surface net tows were conducted during the summers of 2006 and 2007 using a 50 cm diameter, 63 μm mesh net along a 50 m transect at each site. The volume of water clearing the net was quantified using a mechanical flow meter (General Oceanics). Nets which had been towed were stored on ice to maintain the structural stability of coral eggs and planulae (Hodgson 1985) for no more than 2 h before analysis. Coral eggs and planulae were identified to genus and counted; the dominant zooplankton and phytoplankton groups were also noted, but not quantified. Samples were filtered onto pre-weighed 47 mm diameter glass-fiber filters (GF/F) (Whatman), dried at 60°C for 3 d, and

subsequently weighed to determine plankton mass. Samples for chlorophyll *a* (chl *a*) (140 ml) were filtered onto 25 mm diameter GF/F filters (Whatman) and stored at -80°C until extraction in 100% acetone and measurement of fluorescence using a Turner 10-AU fluorometer (Turner Designs). Chl *a* concentrations were calculated using standard methods (Strickland & Parsons 1972).

Carbon and nitrogen content of coral eggs. To estimate the carbon content of a single *Montipora capitata* egg, a calculation was performed based on data from the seawater TOC measurements. A sample analyzed for TOC included visible eggs, and contained $221.7\ \mu\text{M}$ of TOC. The mean concentration of TOC from seawater measurements collected 2 h prior to and following this contaminated sample was $78.3\ \mu\text{M}$. This value was removed from the concentration in the contaminated sample, leaving $143.4\ \mu\text{mol}$ of TOC originating from the coral eggs. The exact number of eggs in the sample was not counted, but was estimated to be 3 to 5. We used this range to approximate a TOC content of 28.7 to $47.8\ \mu\text{mol C egg}^{-1}$. Based on a *M. capitata* egg C:N ratio of 12:1 (J. Padillo-Gamino pers. comm.), each egg also contained 2.4 to 4.0 μmol of total N.

T-RFLP analysis of bacterial SSU rRNA genes. Approximately 1 l of seawater was filtered through a GF/A glass microfiber membrane pre-filter (1.6 μm nominal pore size, Whatman International) followed by a 13 mm diameter, 0.2 μm pore-sized polyethersulfone membrane (Supor 200, Pall Gelman). Filters were stored at -80°C in DNA lysis buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 1.2% v/v Triton X100) (Suzuki et al. 2001). Genomic DNA was extracted from the 0.2 μm pore-sized polyethersulfone membranes using a modified version of the DNeasy Tissue kit (Qiagen) (Becker et al. 2007), and quantified using a PicoGreen fluorescent assay (Invitrogen) on a SpectraMax M2 plate reader (Molecular Devices). For terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu et al. 1997), bacterioplankton small-subunit (SSU) ribosomal RNA (rRNA) genes (including those of heterotrophic bacteria, cyanobacteria, and eukaryotic plastids) were first amplified via the polymerase chain reaction (PCR) using the bacterial primers 27F-B-FAM (5'-AGRGTTYGATYMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3'), with 'FAM' indicating 5' end-labeling of the 27F-B primer with the 6-carboxyfluorescein (FAM) fluorochrome. Each 50 μl PCR reaction contained 0.625 U of PicoMaxx high-fidelity DNA polymerase (Stratagene), 1 \times PicoMaxx reaction buffer, 200 μM of each deoxynucleoside triphosphate (dNTP), 200 nM of each primer and 10 ng of environmental genomic DNA template. After an initial denaturation step at 95°C for 5 min, the reaction conditions were: 24 cycles of 95°C denaturation for 30 s, 55°C annealing for 1 min, and 72°C extension for 2 min, concluding with an extension at

72°C for 20 min. The reactions were performed in a MyCycler Personal Thermal Cycler (Bio-Rad Laboratories). Amplification products were purified using the QIAquick PCR Purification Kit (Qiagen), and subsequently restricted in a 10 μl reaction containing 100 ng of purified amplification product, 2 μg of bovine serum albumin (BSA), 1 \times enzymatic reaction buffer, and 5 units of HaeIII restriction endonuclease (Promega) for 7 h at 37°C . Restriction digests were purified using the QIAquick Nucleotide Removal Kit (Qiagen), and 30 ng μl^{-1} of each product was subsequently electrophoresed on an ABI 3100 Genetic Analyzer (Applied Biosystems). GeneMapper software (Applied Biosystems) was used to estimate the length (in base pairs) and relative abundance of the resulting fragments. Operational taxonomic units (OTUs) were identified as terminal restriction fragments (T-RFs) detected between 33 and 550 bp in length. To account for small differences in the amount of DNA loaded on the ABI 3100, data were normalized by excluding peaks that contributed to $<0.05\%$ of the total peak area for each sample (Sait et al. 2003). An average of 2 peaks were removed from the samples, with a minimum of no peaks and a maximum of 19 peaks removed. An in-house dataset linking cloned bacterial and plastid SSU rRNA gene sequences to T-RFLP profiles from Kaneohe Bay seawater was analyzed in order to putatively identify T-RFs of interest (Yeo et al. unpubl.).

Statistical analyses. Normalized T-RFLP data were analyzed using PC-ORD software (MjM Software Design). Multi-response permutation procedure (MRPP) analysis was employed to test for significant differences in T-RFLP bacterioplankton communities between sites (McCune & Grace 2002). Species indicator analysis was used to identify T-RFs which were significantly different between parameters (Dufrene & Legendre 1997, McCune & Grace 2002). Non-metric multidimensional scaling (NMS) analysis was utilized to explore relationships between individual T-RFLP samples, and was conducted using the Sorenson (Bray-Curtis) distance measure with slow and thorough autopilot criteria (McCune & Grace 2002, Fierer & Jackson 2006). Mantel tests using Sorenson (Bray-Curtis) distance measures and Monte Carlo simulations (1000 randomized runs) were employed to test the relationship between quantitative abiotic and biotic parameters against the T-RFLP profiles.

RESULTS

Non-spawn reef flat and lagoon characteristics

While both the reef flat and lagoon environments investigated in this study contain broadcast-spawning reef-building corals (primarily *Porites compressa* and

Montipora capitata), they differ in their general flow regime. The reef flat site, located off the south coast of Coconut Island (Fig. 1B), experiences high surface water flow driven by the near-constant northeast trade winds (Bathen 1968, Ostrander et al. 2008). The lagoon site is semi-enclosed, sheltered from the winds, and experiences comparatively lower surface flow (Fig. 1). However, both sites exhibited variable water characteristics during non-spawning conditions (Table 1). For example, inorganic nutrient concentrations varied by 1 to 2 orders of magnitude at both sites, while the concentration of chl *a*, rate of BCP, cellular abundance of heterotrophic bacteria, *Synechococcus* and picoeukaryotes, and plankton biomass varied 2- to 3-fold (Table 1). *Synechococcus* and *Prochlorococcus* cells were distinguishable with the flow cytometry methods employed in this study, though no *Prochlorococcus* cells were detected at either site.

Statistical analyses revealed that the mean concentrations of several inorganic nutrients, TOC, pH, rates of BCP, and the cellular abundance of heterotrophic bacteria, *Synechococcus* and picoeukaryotes were significantly different between the reef flat and lagoon (1-way ANOVA, $p < 0.05$; Table 1). In particular, mean values for TOC and the cellular abundance of heterotrophic bacteria, *Synechococcus* and picoeukaryotes

were elevated at the reef flat sampling site, and BCP and inorganic nutrients were elevated in the lagoon.

In the lagoon, the cellular abundance of heterotrophic bacteria, *Synechococcus* and picoeukaryotes were significantly correlated to each other ($p < 0.02$, $n = 45$). Significant correlations were also observed for the cellular abundance of *Synechococcus* and concentrations of PO_4^{3-} , $\text{NO}_3^- + \text{NO}_2^-$, NO_2^- and NH_4^+ ($p < 0.04$, $n = 45$) and the cellular abundance of picoeukaryotes and NO_2^- and NH_4^+ ($p < 0.01$, $n = 45$); no relationship was found between inorganic nutrients and the cellular abundance of heterotrophic bacteria. However, both heterotrophic bacteria and picoeukaryote abundances were correlated with chl *a* concentrations ($p < 0.02$, $n = 45$).

Similar to the lagoon sampling site, the cellular abundance of heterotrophic bacteria, *Synechococcus* and picoeukaryotes were also significantly correlated to each other on the reef flat ($p < 0.05$, $n = 40$). However, relationships between cellular abundances and inorganic nutrients were less apparent compared to the lagoon, as only the abundance of *Synechococcus* and NO_2^- concentrations were significantly correlated ($p < 0.04$, $n = 40$). Similar to the lagoon, the cellular abundance of heterotrophic bacteria at the reef flat was correlated with concentrations of chl *a* ($p < 0.02$, $n = 40$).

Table 1. Comparison of surface water characteristics (mean values and ranges) for reef flat and lagoon sites in Kaneohe Bay, Hawaii, USA, during non-spawn conditions ($n = 40$ to 45, with exceptions noted). *Site characteristics are significantly different (1-way ANOVA, $p < 0.05$)

Parameter	Reef flat non-spawn ^a	Lagoon non-spawn
Temperature (°C)	26.24 (22.95–27.78)	26.13 (22.75–28.44)
Salinity (psu)	34.63 (28.24–36.43)	34.57 (29.34–36.72)
pH*	7.97 (7.82–8.13)	7.91 (7.80–8.01)
$\text{NO}_3^- + \text{NO}_2^-$ (μM)*	0.601 (0.010–8.050)	0.917 (0.024–3.660)
NO_2^- (μM)	0.104 (0.010–0.221)	0.063 (0.020–0.320)
NH_4^+ (μM)*	0.432 (0.017–1.053)	0.207 (0.015–0.910)
PO_4^{3-} (μM)	0.152 (0.061–1.340)	0.169 (0.057–0.634)
Silicate (μM)	9.06 (0.74–19.35)	9.43 (1.91–20.70)
TOC (μM) ^{b*}	78.59 (76.77–79.51)	72.64 (70.49–74.98)
BCP (μg C l ⁻¹ h ⁻¹) ^{c*}	0.1287 (0.0647–0.2557)	0.1901 (0.0603–0.4280)
Heterotrophic bacteria (×10 ⁶ cells ml ⁻¹)*	1.42 (0.88–2.33)	0.96 (0.52–1.80)
<i>Synechococcus</i> (×10 ⁵ cells ml ⁻¹)*	2.09 (0.66–4.93)	0.99 (0.21–3.09)
Picoeukaryotes (×10 ⁴ cells ml ⁻¹)*	2.60 (0.85–5.04)	1.44 (0.37–3.02)
Chlorophyll <i>a</i> (μg l ⁻¹)	0.97 (0.24–1.78)	1.09 (0.26–4.65)
Plankton biomass (mg dry weight m ⁻³) ^c	69 (10–140)	66 (7–103)

^aNon-spawn samplings from January 2006 to July 2007 obtained at least 1 wk after a spawning event
^b $n = 6$
^c $n = 16$

Bacterioplankton diversity during non-spawn conditions

T-RFLP fingerprinting of the bacterioplankton communities revealed a total of 242 T-RFs of unique length for the entire, combined dataset. The reef flat and lagoon sites averaged 62 and 59 T-RFs per sample, respectively (range 22 to 101), and 10 T-RFs were present in all samples irrespective of site (Table 2).

Non-metric multidimensional scaling analysis uncovered temporal variability in the composition of the lagoon and reef flat bacterioplankton communities (data not shown), as well as a partitioning between samples from the lagoon and reef flat sites (Fig. 2A). An MRPP test found significant differences between the lagoon and reef flat bacterial communities ($p < 0.0001$, $A = 0.098$).

Mantel tests were used to examine relationships between bacterioplankton community structure based on T-RFLP and other measured environmental variables; this revealed that salinity,

pH, silicate and the cellular abundance of *Synechococcus* and picoeukaryotes were significantly related to the structure of the bacterioplankton community at the reef flat (Table 3). NMS analysis of the T-RFLP profiles of bacterioplankton community structure from the reef flat in relation to the factors identified via Mantel tests demonstrated that a few profiles were related to increasing concentrations of silicate, *Synechococcus* or picoeukaryotes, but most were not directly related to these autotrophy-related parameters (Fig. 2B). At the lagoon site, abundances of chl *a*, *Synechococcus*, picoeukaryotes, heterotrophic bacteria, $\text{NO}_3^- + \text{NO}_2^-$, silicate and NO_2^- were significantly related to the composition of the bacterioplankton community (Table 3). A comparison of the lagoon T-RFLP profiles and factors identified via Mantel tests revealed that only a few profiles were related to increasing values of these identified factors, and the majority of the profiles were not directly associated with any of these parameters (Fig. 2C).

Table 2. Summary of terminal restriction fragments (T-RFs) either ubiquitous in all reef flat and lagoon samples, or correlating to coral spawning. (u): T-RFs that are ubiquitously distributed throughout all samples analyzed in this study; +, -: T-RFs that are either positively (+) or negatively (-) correlated to the presence of coral eggs; nd: a T-R with a phylogenetic affiliation not determined

T-RF	Putative phylogenetic affiliation	Reef flat	Lagoon
34	Mixed (<i>Alphaproteobacteria</i> , <i>Rhodobacterales</i> ; <i>Gammaproteobacteria</i> ; <i>Bacteroidetes</i>)	(u)	(u)
61	<i>Alphaproteobacteria</i> , SAR11 subgroup V	(u)	(u)
112	<i>Alphaproteobacteria</i> , SAR11 subgroup IA	(u)	(u)
135	<i>Cyanobacteria</i> , marine <i>Synechococcus</i>	+ (u)	(u)
170	<i>Verrucomicrobia</i> , <i>Opitutae</i> lineage		-
186	<i>Gammaproteobacteria</i> , SAR86 subgroups I & II		-
189	nd		+
190	nd	(u)	(u)
191	<i>Alphaproteobacteria</i> , SAR116 subgroups IV & VII	(u)	(u)
201	nd	+	+
203	<i>Alphaproteobacteria</i> , OCS124 lineage		-
206	nd	-	
219	nd		+
222	<i>Betaproteobacteria</i> , OM43	+	+
224	<i>Alphaproteobacteria</i> , SAR116 subgroup II		+
226	<i>Alphaproteobacteria</i> , SAR11 subgroup IB	(u)	(u)
229	Mixed (Candidate phylum SAR406/ Marine Group A; <i>Verrucomicrobia</i>)	-	-
254	<i>Gammaproteobacteria</i> , HTCC2188	-	
276	nd	+	
283	nd	-	
288	<i>Cyanobacteria</i> , marine <i>Synechococcus</i>		+
289	<i>Alphaproteobacteria</i> , SAR11 subgroups II & III	(u)	(u)
298	nd		+
314	nd		+
323	<i>Cyanobacteria</i> , prasinophyte plastid	-	
328	<i>Actinobacteria</i> , marine <i>Actinobacteria</i> clade	(u)	+ (u)
371	nd	+	
379	<i>Cyanobacteria</i> , diatom plastid		+
385	<i>Cyanobacteria</i> , prasinophyte plastid	(u)	(u)

Coral spawning events

The presence of coral eggs in surface waters of Kaneohe Bay coincided with the phases of the new moon and the full moon (Fig. 3). Full-moon spawning events were primarily composed of eggs from *Porites compressa*, while new-moon events were dominated by eggs from *Montipora capitata*. The new-moon spawnings in June and July were comparably larger than full-moon spawnings. Overall, the abundance of coral eggs at the sites varied widely (0 to 2088 eggs m^{-3}) (Fig. 3).

A diverse community of plankton was identified within the towed nets, with both sample sites harboring multiple species of copepods, fish larvae and chaetognaths. Large diatoms (primarily *Chaetoceros* spp.) were frequently recovered from nets towed at both sites. Occasionally, radiolarians and appendicularians were present in the nets, while oyster larvae were routinely recovered from the lagoon. Elevated plankton biomass was generally (but not significantly) related to coral egg abundance at both sites (Fig. 3).

From a net tow conducted immediately following a large *Montipora capitata* spawning event on July 26, 2006, at 20:00 h, it was estimated that surface waters of the lagoon site contained over 70 000 coral eggs m^{-2} . Measurements of the carbon and nitrogen content of single eggs were not directly conducted, but a calculation estimated that *M. capitata* released 2009 to 3346 mmol of particulate organic carbon and 168 to 280 mmol of particulate nitrogen m^{-2} into the lagoon that evening.

Microbial response to coral spawning events

Throughout the summer, the concentration of chl *a* and the cellular abundance of heterotrophic bacteria and *Synechococcus* fluctuated 1.5- to 2-fold, while the cellular abundance of picoeukaryotes varied up to 3- to 5-fold at both sampling sites (Fig. 4). Following coral spawning, the cellular abundance of heterotrophic bacteria, *Synechococcus* and picoeukaryotes, as well as concentrations of chl *a*, did not consistently increase or decrease beyond pre-spawn values at either site (Fig. 4). Additionally, when post-spawn

increases in microbial cellular abundance were observed, they did not exceed the variability of these parameters observed outside of spawning events. On the reef flat, the rate of BCP and the concentration of TOC did not change following *Montipora capitata* spawning events (Fig. 5A). In the lagoon, BCP increased following the July 16, 2007, *M. capitata* spawning event, though this rate did not exceed pre-spawn values (Fig. 5B). Following the largest spawning night in July 2007, the lagoon harbored elevated concentrations of TOC (Fig. 5B), but these levels did not persist over subsequent nights of spawning.

The composition of the reef flat bacterioplankton community was significantly correlated to the presence of coral eggs (MRPP test, $A = 0.031$, $p < 0.01$) but not with the abundance of coral eggs (Mantel test, $r = 0.039$, $p = 0.19$). T-RFs of 135, 201, 222, 276 and 371 bp were positively correlated, and T-RFs of 206, 229, 254, 283 and 323 bp were negatively correlated with the presence of coral eggs at the reef flat (Table 2). However, the positively correlated T-RFs were found to increase only slightly in relative abundance (<2%) following the largest coral spawning event investigated (July 24 to 27, 2006) (Fig. 6A). The 135 and 222 bp T-RFs comprised the largest component of the spawn-affected bacterioplankton community, though the relative abundance of each was <5% of the total bacterioplankton community (Fig. 6A). The composition of the lagoon bacterioplankton community was significantly correlated to both the presence and abundance of coral eggs (Mantel test, $r = 0.068$, $p < 0.04$; MRPP test, $A = 0.022$, $p < 0.05$). A species indicator test demonstrated that T-RFs of 189, 201, 219, 222, 224, 288, 298, 314, 328 and 379 bp were positively correlated and T-RFs of 170, 186, 203 and 229 bp were negatively correlated with the presence of coral eggs in the lagoon (Table 2). Similar to the the reef flat, the positively correlated T-RFs increased only slightly (<3%) in relative abundance following a large spawning event, and made up a small (<5%) component of the total bacterioplankton community (Fig. 6B). At both locations, a T-RF of 222 bp increased following spawning, which was putatively identified as corresponding to the OM43 clade of *Betaproteobacteria* (Rappé et al. 1997) (Table 2).

Lagoon diurnal microbial dynamics during spawn and non-spawn conditions

In the lagoon, sampling was conducted every 2 to 3 h for 48 h following the July 2007 *Montipora capitata* spawning (Fig. 7A–D). On July 14, spawning occurred at about 20:00 h, coinciding with a drop in tidal height. From 00:00 to 02:00 h, inorganic nitrogen and phospho-

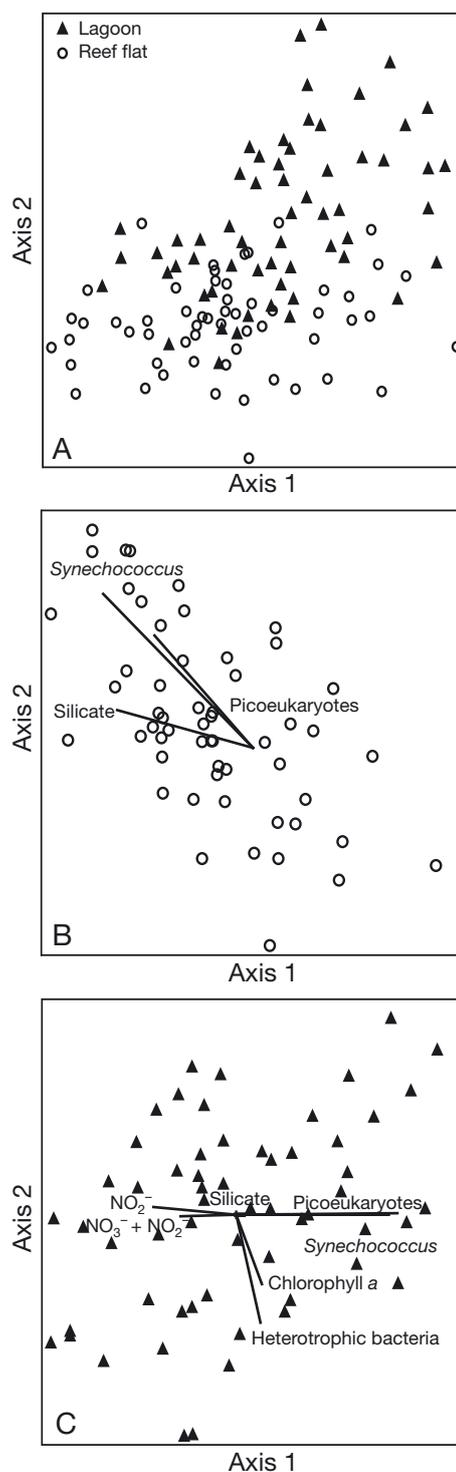


Fig. 2. Non-metric multidimensional scaling analyses of bacterial community structure during non-spawning conditions over the 18 mo survey, based on T-RFLP. (A) A comparison of the reef flat and lagoon site bacterial communities. Each ordinated sample represents an individual sample date. T-RFLP profiles of (B) the reef flat and (C) the lagoon are compared to co-varying factors, with the vector lengths representing increasing values of each parameter. The stress of the 3-dimensional solutions are (A) 0.146, (B) 0.102 and (C) 0.100

Table 3. Relationships between bacterioplankton community T-RFLP profiles and abiotic and biotic factors during non-spawn conditions, from Mantel tests.
*Significant relationship ($p < 0.05$)

Variable	Reef flat (r statistic, p-value)	Lagoon (r statistic, p-value)
Plankton biomass	-0.028, 0.395	-0.092, 0.052
Chlorophyll <i>a</i> concentration	0.104, 0.137	0.130, 0.028*
<i>Synechococcus</i> abundance	0.386, 0.001*	0.303, 0.001*
Picoeukaryote abundance	0.144, 0.040*	0.249, 0.001*
Heterotrophic bacteria abundance	0.014, 0.381	0.110, 0.023*
PO ₄ ³⁻ concentration	0.102, 0.082	0.070, 0.099
NO ₃ ⁻ + NO ₂ ⁻ concentration	0.003, 0.531	0.145, 0.016*
NH ₄ ⁺ concentration	0.101, 0.149	0.084, 0.074
Tidal height	0.048, 0.248	-0.051, 0.241
Temperature	0.034, 0.188	0.074, 0.187
Salinity	-0.076, 0.002*	0.051, 0.233
pH	0.330, 0.001*	0.129, 0.051

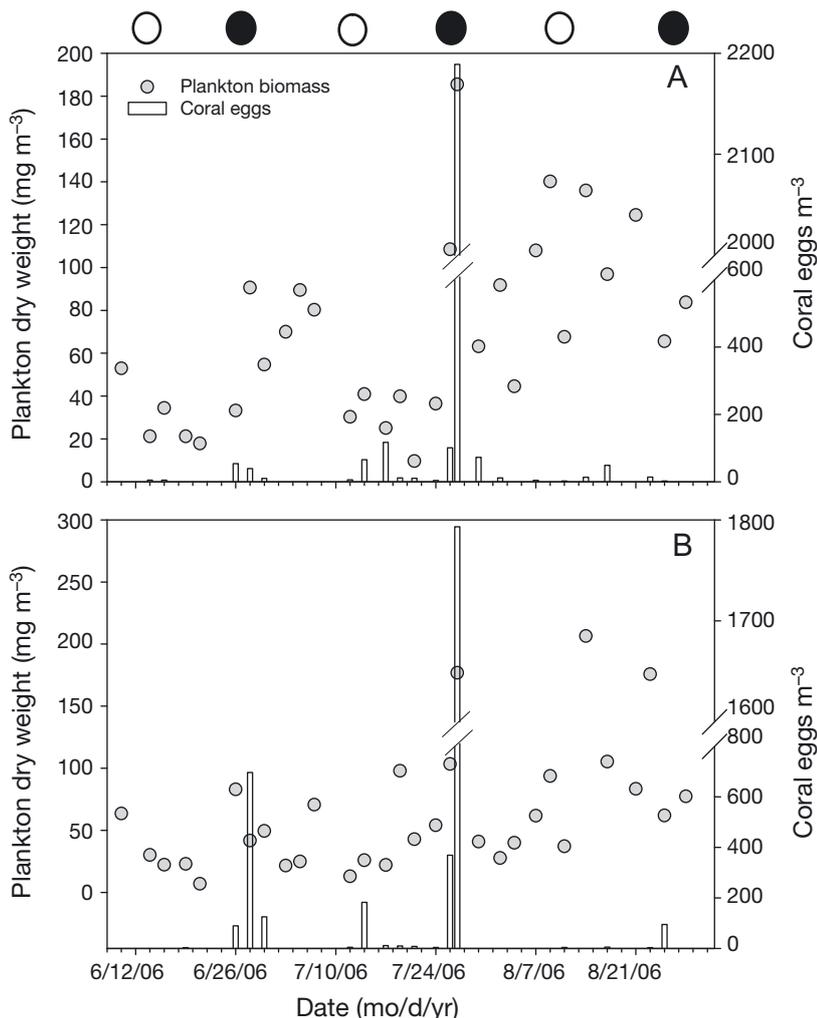


Fig. 3. Abundance of coral eggs and plankton biomass in the surface waters of Kaneohe Bay at the (A) reef flat and (B) lagoon sites during the summer of 2006. Open and closed circles at the top of the panels represent full- and new-moon phases, respectively

rus decreased 4- to 5-fold, which coincided with a 2-fold increase in heterotrophic bacteria and picoeukaryotes and a 3-fold increase in *Synechococcus* cellular abundance (Fig. 7A–C). The concentration of chl *a* remained relatively unchanged throughout this period (Fig. 7B). On the second night, spawning was again associated with a decreasing tide, while concentrations of inorganic nutrients and chl *a* peaked and subsequently declined, coinciding with a 2.5- to 4-fold increase in cellular abundance of heterotrophic bacteria, *Synechococcus* and picoeukaryotes. Rates of BCP varied little throughout the night, but exhibited elevated levels during daylight hours (Fig. 7D). TOC concentrations remained steady throughout the sampling period, with the exception of one sample collected during the second night of spawning which contained $>220 \mu\text{M}$ TOC and visible coral eggs in the sample (Fig. 7D). The concentration of chl *a* was also elevated in this sample (Fig. 7B), possibly due to the contribution of chlorophyll from symbiotic dinoflagellates contained within coral eggs.

Measurements conducted in the lagoon over a period of 24 h during non-spawning conditions (October 2007) also demonstrated temporal fluctuations in inorganic nutrient concentrations and microbial cell abundances at night, but were generally lower in magnitude than observed during the spawning events (Fig. 7E–G). In addition, BCP rates did not change between day and night (Fig. 7H). It should be noted that the tidal fluxes between the diurnal study periods were different: the July new-moon flux was 0.80 m and the October flux was 0.16 m.

DISCUSSION

Pelagic microbial response to coral spawning events

A relationship between the composition of the bacterioplankton community and the presence of spawned coral eggs was observed in both lagoon and reef

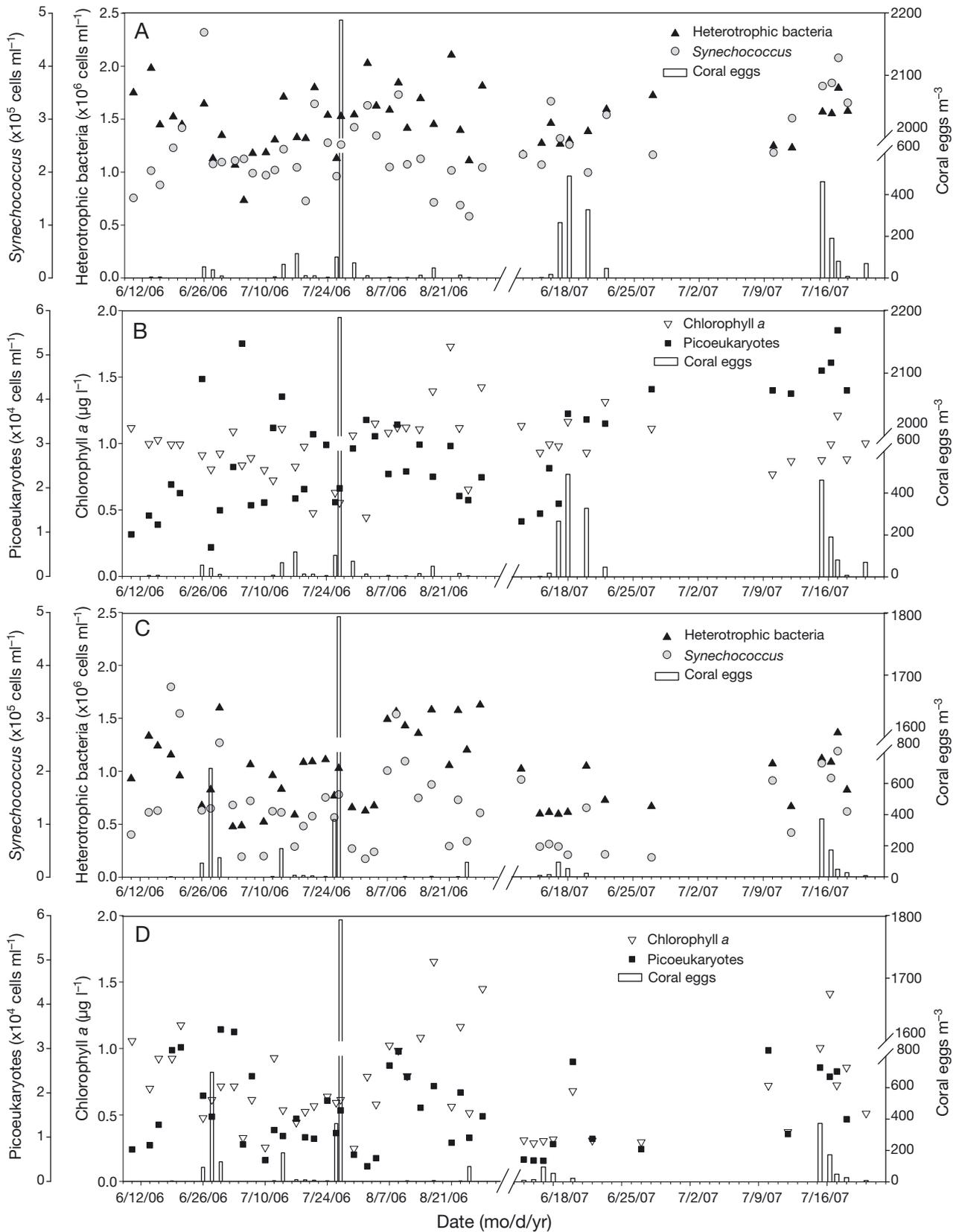


Fig. 4. Temporal abundances of (A,C) heterotrophic bacteria and *Synechococcus* and (B,D) picoeukaryotes and chlorophyll a in relation to coral eggs at the (A,B) reef flat and (C,D) lagoon sites during the summers of 2006 and 2007

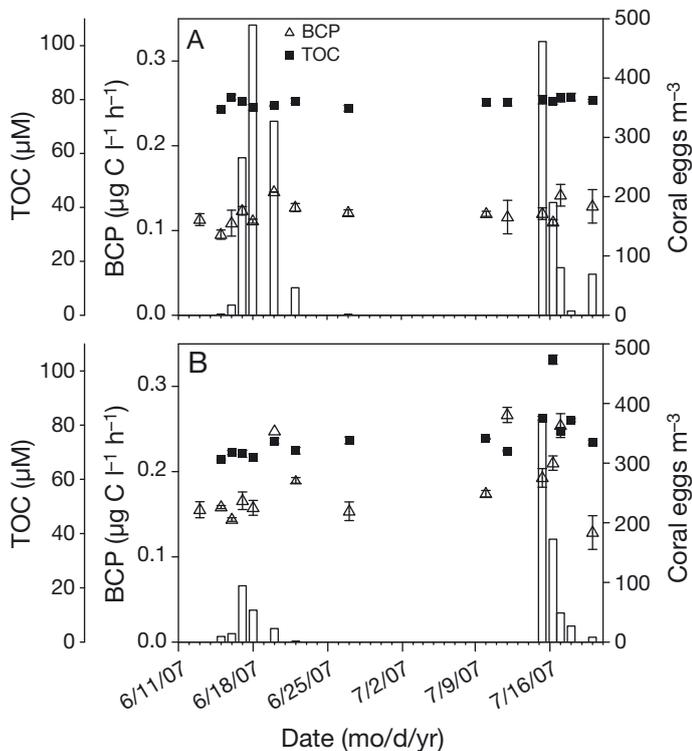


Fig. 5. Comparison of rates of bacterial carbon production (BCP) and total organic carbon (TOC) concentrations measured in the (A) reef flat and (B) lagoon surrounding 2 *Montipora capitata* spawning events. Abundances of coral eggs are shown by the bars. Error bars are \pm SD for triplicate samples

flat sampling sites over the course of 2 summers and multiple spawning events. However, based on T-RFLP, the change in the composition of the bacterioplankton community was only slight, and only a small number of bacterioplankton groups positively correlated to the spawning events. In addition, bacterial production rates did not change in the hours or days following spawning. Thus, only a small percentage of the bacterioplankton community appeared able to utilize organic matter from the coral gametes. Coral eggs comprise 60 to 70% lipids by weight (primarily wax esters) (Arai et al. 1993), resulting in highly skewed, carbon-dominated C:N:P ratios such as 316:16:1 for coral eggs (Eyre et al. 2008). While organic phosphorus sources (including lipids) are readily utilized by marine bacterioplankton (Björkman & Karl 2003), nothing is currently known regarding the lability of dissolved coral lipids as a substrate for growth of heterotrophic bacteria. Surprisingly, in this study we found that one of the major bacterial lineages responding to spawn events was the OM43 clade of *Betaproteobacteria* (Rappé et al. 1997), a group of obligate methylotrophs that can utilize only methanol and a limited number of other 1-carbon organic compounds for growth (Gio-

vannoni et al. 2008). This bacterial group commonly appears in coastal seawater environments of the global ocean, and has been previously found associated with a phytoplankton bloom off the coast of Oregon, USA (Morris et al. 2006). The simple nature and limited scope of substrates known to promote the growth of OM43 cells indicate that it is highly unlikely that they are utilizing coral lipids directly, and the ultimate source of the substrates promoting their growth after coral spawning events remains unknown.

Another factor that appears to contribute to the relative lack of a response from the microbial community to coral spawning is the rapid advection or sinking of particulate material prior to dissolution and utilization by planktonic microorganisms. In this study, we estimated that 2009 mmol m⁻² of egg-associated carbon was released into the lagoon during one spawning night. However, seawater TOC concentrations in the lagoon just hours following spawning were not elevated above pre-spawned levels, with the exception of one sample containing visible, intact eggs. We conclude that the spawned material is patchily distributed in particulate form in the surface waters of the lagoon until it sinks or is advected out of the system. Interestingly, the concentrations of TOC at both sites during spawn and non-spawn conditions were similar in magnitude to dissolved organic carbon concentrations in the North Pacific Subtropical Gyre, as well as pristine coral reef atolls (Church et al. 2002, Dinsdale et al. 2008), and were less than dissolved organic carbon concentrations found at more anthropogenically impacted coral reef environments (Garren et al. 2008, Rochelle-Newall et al. 2008). While Kaneohe Bay routinely receives input from streams originating from the surrounding urban terrain, the stable and relatively low concentrations of TOC present at the lagoon and reef flat sampling sites indicate that the terrestrial input of organic carbon may not reach all environments within Kaneohe Bay, or else it is rapidly diluted or advected after highly episodic input (i.e. storm runoff) events.

Coral spawning events in the Great Barrier Reef have recently been shown to elicit significant pelagic microbial responses that are primarily attributed to photoautotrophic microorganisms (Eyre et al. 2008, Glud et al. 2008, Patten et al. 2008, Wild et al. 2008). These phytoplankton blooms result in primary production rates that are significantly elevated over respiration, indicating that phototrophic nutrient recycling dominates the planktonic response to mass coral spawning, rather than heterotrophic utilization of spawned organic carbon (Eyre et al. 2008, Glud et al. 2008). Regardless, an increase in heterotrophic microbial activity following a spawning event in the Great Barrier Reef was inferred from increased pelagic oxygen consumption (Wild et al. 2008) and a 2-fold

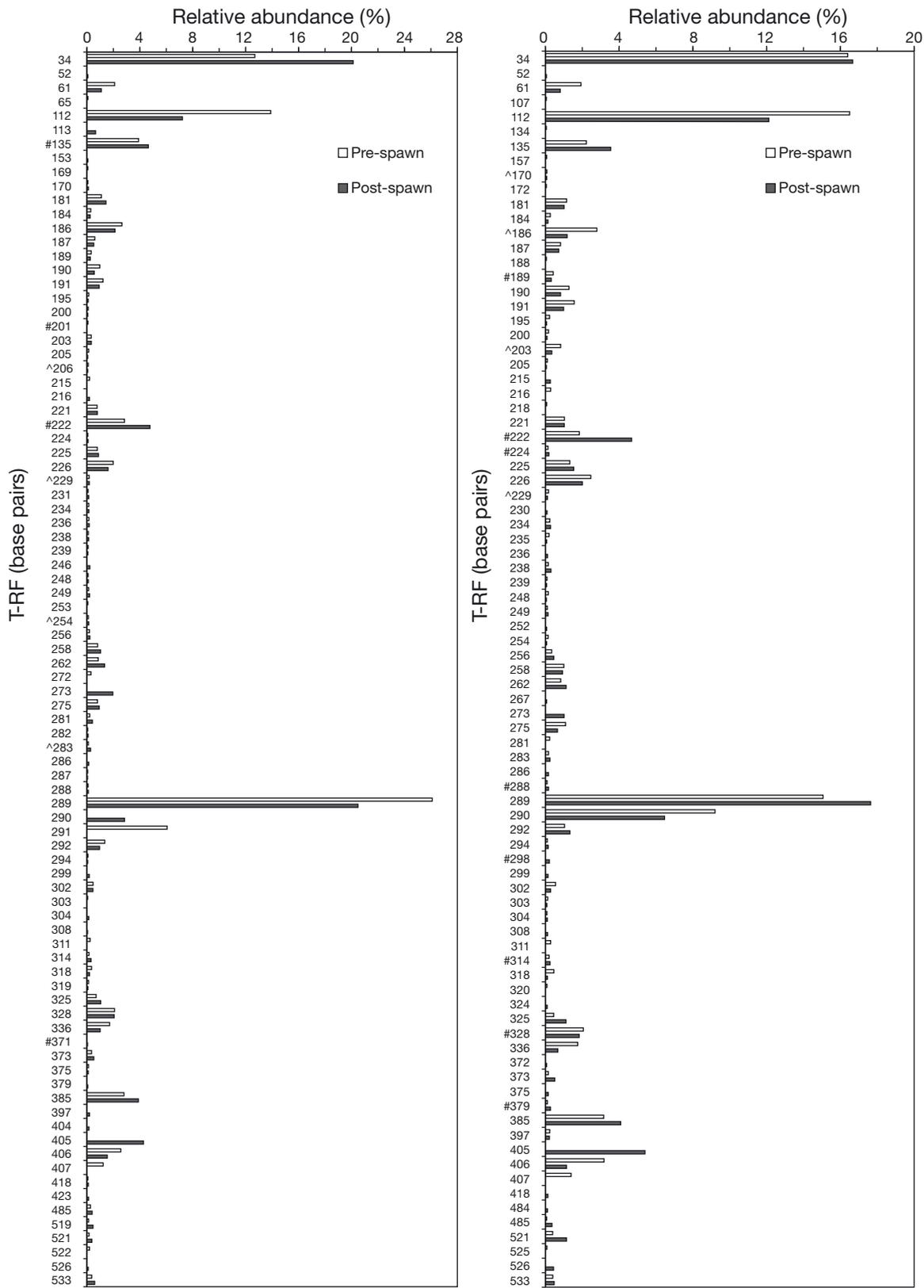


Fig. 6. Relative abundances of terminal restriction fragments (T-RFs) measured prior to (July 24, 2006) and immediately following (July 27, 2006) a large *Montipora capitata* spawning event at the (A) reef flat and (B) lagoon sites. T-RFs that were positively (#) and negatively (^) correlate to the presence of eggs at each site are indicated

increase in microbial cell abundance (Patten et al. 2008). In the present study, we did not observe enhanced heterotrophic productivity, cellular abundances of planktonic heterotrophic bacteria or autotrophs, or chl *a* concentrations following the multiple spawning events, and only very minor changes in

bacterioplankton community structure were detected. While large, rapid variations in microbial cell abundances, nutrients and chl *a* concentrations were observed during high frequency, diurnal sampling in the Kaneohe Bay lagoon, no systematic microbial response was detected during post-spawning sam-

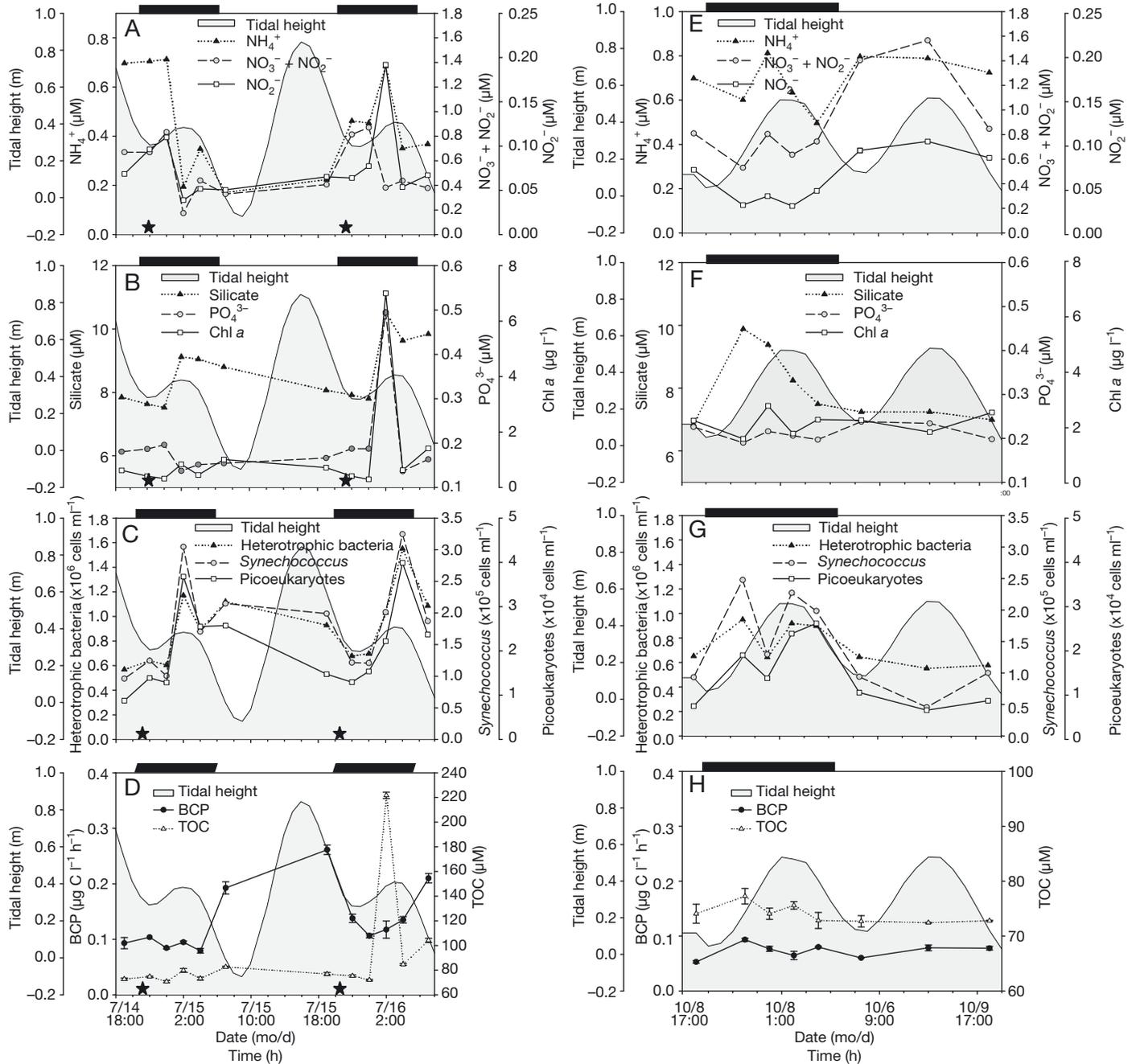


Fig. 7. (A–D) Comparison of diurnal trends of inorganic nutrients, cellular abundances, chlorophyll *a*, carbon production rates, organic carbon and tidal height in the lagoon following *Montipora capitata* spawning in July 2007, and (E–H) during non-spawning conditions in October 2007. (A,E) Concentrations of dissolved inorganic nutrients $\text{NO}_3^- + \text{NO}_2^-$, NO_2^- , and NH_4^+ . (B,F) Concentrations of PO_4^{3-} , silicate and chlorophyll *a*. (C,G) Abundances of heterotrophic bacteria, *Synechococcus* and picoeukaryotes. (D,H) Rates of bacterial carbon production (BCP) compared to concentrations of total organic carbon (TOC). The dark bars at the top of the panels represent nighttime, and the stars indicate *M. capitata* spawning. Error bars are \pm SD for triplicate samples

plings in 2006 and 2007, and thus these observations are probably attributable to circumstances other than spawning.

Several factors may contribute to the differences in microbial response between previous studies from the Great Barrier Reef and the present study from Kaneohe Bay, Hawaii. For example, the magnitude of spawning likely contributes to the differential observations between studies: the number of synchronous spawning coral species is considerably higher in the Great Barrier Reef compared to Kaneohe Bay (Hodgson 1985, Babcock 1986). A second factor is that the present study characterized the natural variability in microbial communities during non-spawn conditions, and considered the impact of tides. Most large-scale spawning events coincide with extreme new- and full-moon tides, and it is possible that observations of microbial responses attributed to spawning may also be triggered by nutrient and biomass delivery from tides. A third factor to consider is that rates of bacterioplankton production in coastal reefs have been shown to fluctuate more over a single day than from more temporally spaced measurements (Moriarty et al. 1985). Defining normal community variability in temporally dynamic coastal microbial communities is undoubtedly important for understanding how communities respond to episodic nutrient and organic matter loading events.

Diurnal measurements conducted in the lagoon sampling site following *Montipora capitata* spawning captured temporally rapid changes in dissolved inorganic nutrient concentrations and cellular abundances of heterotrophic bacteria, *Synechococcus* and picoeukaryotes. However, it is difficult to reconcile these complex dynamics based on *in situ* biological processes alone. For example, the increase in microbial standing stocks did not coincide with elevated rates of bacterial production, and thus we suspect that the stock increases are probably not attributable to new cellular growth from the spawned material. We believe that these fluctuations may be caused by mixing of the shallow water column and the delivery of new but less productive cellular biomass into the lagoon due to the relatively large new-moon tides. During non-spawn conditions, the lagoon harbors lower cellular abundances of picoeukaryotes, *Synechococcus* and heterotrophic bacteria when compared to the reef flat, but maintains a higher rate of bacterial production. Though speculative, it is plausible that a tidally delivered flux of water from the reef flat environment into the lagoon may contribute to an increase in biomass with little measurable impact on bacterial production.

Interestingly, over the course of diurnal sampling we observed elevated rates of bacterial production during daylight hours, providing further evidence that coral

spawn products were not directly eliciting a response by pelagic heterotrophic bacteria. Previous studies investigating the effect of light on heterotrophic bacterial production in seawater have revealed that planktonic marine bacteria respond significantly to the availability of light, due to photoheterotrophic bacterial production or the presence of photosynthetically produced or photodynamically transformed dissolved organic carbon sources (Norrman et al. 1995, Aas et al. 1996, Moran & Zepp 2000, Moran et al. 2001, Church et al. 2004). While chl *a* concentrations and the abundance of picoeukaryotes were generally not elevated above typical non-spawn levels during the lagoon diurnal measurements, it is possible that the observed daytime increase in bacterial production was caused by heterotrophic microorganisms utilizing photosynthetically produced dissolved organic carbon. Correlations between concentrations of chl *a*, abundances of heterotrophic bacteria and picoeukaryotes and the composition of the bacterioplankton community were observed in the lagoon under non-spawning conditions, indicating a consistent coupling of phytoplankton and bacterioplankton dynamics in this environment.

Differential lagoon and reef flat microbial dynamics

Pelagic coral reef environments are affected by a number of biological, physical and geochemical processes. The reef flat and lagoon microbial communities investigated in this study appear to be controlled by dissimilar processes, in spite of their close proximity. Bacterioplankton community structure differed between the two environments, and lower cell abundances of heterotrophic bacteria but higher rates of bacterial production were observed in the lagoon compared to the reef flat. These results suggest that the heterotrophic microorganisms in the lagoon are more active than their counterparts on the reef flat. The main physical difference between the lagoon and reef flat locations is the mixing regime (Bathen 1968). Turbulence and mixing rate were not directly measured during this study, but it is generally thought that increases in either can stimulate production and growth of phytoplankton communities (Peters et al. 2002, Pinhassi et al. 2004, Guadayol et al. 2009). In contrast, stagnant water has been related to higher rates of heterotrophic bacterial production due to the formation of nutrient-rich patches around particles or phytoplankton (Moeseneder & Herndl 1995). We hypothesize that tidal flow and turbulence are important factors controlling bacterioplankton dynamics in this study, and future research is necessary to further understand this connection.

The identification of bacterioplankton communities that differ in composition between 2 geographically close environments may have implications for microbial food web dynamics, reef ecosystem functioning and the health of corals. For example, distinct differences in the composition of the microbial community have been observed in anthropogenically impacted reef areas that also harbor greater numbers of diseased corals or decreased coral recruitment (Villanueva et al. 2005, Dinsdale et al. 2008, Garren et al. 2008). The lagoon sampled in this study contains a number of corals with symptoms of disease (A. Apprill pers. obs.). It is possible that the unique microbial community present in the lagoon is one of the factors contributing to these disease symptoms, either by hosting pathogenic or opportunistic microorganisms, or by lacking microorganisms which play beneficial roles in the reef microbial food web (Rohwer & Kelley 2004).

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