

**Marine Managed Areas Project  
Advanced Cross-Site Comparison of Ecosystem Health: Coral Reef Habitats**

**Final Report – June 2008**

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**OBJECTIVE**

The primary objective of this activity is to develop a next-generation diagnostic system for comparing ecosystem health for reefs around the world that offers improvements upon current approaches such as the Global Coral Reef Monitoring Network, Atlantic and Gulf Rapid Reef Assessment (AGRRA) and the “scorecard” for the Healthy Reefs Initiative. This system will include representatives of all living organisms in the ecosystem, from microbes to sharks, which will provide a diagnosis of overall current ecosystem health.

**DELIVERABLES**

- 1) A set of indicators and the minimum amount of data needed to quantify the health of a reef ecosystem anywhere in the world.
- 2) A technical description of the protocols and how to collect and analyze data to calculate the indicators

**FINAL RESULTS**

**Baselines and degradation of coral reefs in the Northern Line Islands**

Effective conservation requires rigorous baselines of pristine conditions to assess the impacts of human activities and to evaluate the efficacy of management. Most coral reefs are moderately to severely degraded by local human activities such as fishing and pollution as well as global change, hence it is difficult to separate local from global effects. To this end, we surveyed coral reefs on uninhabited atolls in the northern Line Islands (Fig. 1) to provide a baseline of reef community structure, and on increasingly populated atolls to document changes associated with human activities. We found that top predators and reef-building organisms dominated unpopulated Kingman and Palmyra, while small planktivorous fishes and fleshy algae dominated the populated atolls of Tabuaeran and Kiritimati (Figs. 2-3). Sharks and other top

predators overwhelmed the fish assemblages on Kingman and Palmyra so that the biomass pyramid was inverted (top-heavy). In contrast, the biomass pyramid at Tabuaeran and Kiritimati exhibited the typical bottom-heavy pattern. Reefs without people exhibited less coral disease and greater coral recruitment relative to more inhabited reefs (Fig. 4). Thus, protection from overfishing and pollution appears to increase the resilience of reef ecosystems to the effects of global warming.

Citation: Sandin et al (2008) Baselines and Degradation of Coral Reefs in the Northern Line Islands. PLoS ONE 3(2): e1548.

### **Microbial ecology of four coral atolls in the Northern Line Islands**

Microbes are key players in both healthy and degraded coral reefs. A combination of metagenomics, microscopy, culturing, and water chemistry were used to characterize microbial communities on four coral atolls in the Northern Line Islands, central Pacific. Kingman, a small uninhabited atoll which lies most northerly in the chain, had microbial and water chemistry characteristic of an open ocean ecosystem. On this atoll the microbial community was equally divided between autotrophs (mostly *Prochlorococcus* spp.) and heterotrophs. In contrast, Kiritimati, a large and populated (,5500 people) atoll, which is most southerly in the chain, had microbial and water chemistry characteristic of a near-shore environment. On Kiritimati, there were 10 times more microbial cells and virus-like particles in the water column and these microbes were dominated by heterotrophs, including a large percentage of potential pathogens (Fig. 5). Culturable *Vibrios* were common only on Kiritimati. The benthic community on Kiritimati had the highest prevalence of coral disease and lowest coral cover. The middle atolls, Palmyra and Tabuaeran, had intermediate densities of microbes and viruses and higher percentages of autotrophic microbes than either Kingman or Kiritimati. The differences in microbial communities across atolls could reflect variation in 1) oceanographic and/or hydrographic conditions or 2) human impacts associated with land-use and fishing. The fact that historically Kingman and Kiritimati did not differ strongly in their fish or benthic communities (both had large numbers of sharks and high coral cover) suggest an anthropogenic component in the differences in the microbial communities. Kingman is one of the world's most pristine coral reefs, and this dataset should serve as a baseline for future studies of coral reef microbes. Obtaining the microbial data set, from atolls is particularly important given the association of microbes in the ongoing degradation of coral reef ecosystems worldwide.

Citation: Dinsdale EA, Pantos O, Smriga S, Edwards RA, Angly F, et al (2008) Microbial Ecology of Four Coral Atolls in the Northern Line Islands. PLoS ONE 3(2): e1584

### **Deliverable 1: Integrating the coral reef community: what are the best indicators?**

A principal components analysis (PCA) showed that there is a gradient from a reef dominated by top predators (piscivorous fishes), hard corals and crustose coralline algae to a reef dominated by turf and erect algae, secondary consumers (carnivorous fishes) and microbes (Fig. 6). Because of the complexity at the community level, we also performed PCAs on fishes and benthic communities separately, which confirmed the clear trends. All of these multivariate analyses confirm that:

- 1) Increasing fishing pressure causes a decline in the biomass of piscivorous fishes (top predators). Top predator biomass is significantly correlated with total fish biomass (Friedlander & DeMartini 2002, Newman et al. 2006, Knowlton & Jackson 2008, Sandin et al. 2008). Because many degraded reefs do not have large piscivorous as

top predators, **total fish biomass is the best single indicator of the health of reef fish communities** for large-scale comparisons.

- 2) Increasing human activities is associated with a shift from communities dominated by hard corals and crustose coralline algae (CCA) to communities dominated by turf and erect algae. Coral cover is an indicator of the current health of the coral community; CCA cover is an indicator of the potential for recovery of the coral assemblage after warming events since coral recruitment was significantly correlated with CCA cover. **The cover of hard corals + CCA is a good indicator of the current health of benthic communities.**
- 3) Increasing human population is associated with an increase in the abundance of microbes and microbial activity. In turn, microbial activity is related to coral disease (Smith et al. 2006). Microbial abundance is also positively related to viral abundance and a shift from autotrophy to heterotrophy in the microbial community (Dinsdale et al. 2008). **Microbial abundance is the single best indicator of the health of microbial communities and risk of coral disease.**

Total fish biomass is correlated with most major changes in reef communities on the Line Islands, and one could consider using fish biomass as the only indicator of health. However, in other places where there are no pristine reefs left, reef fishes and benthic communities are decoupled in terms of their position within a gradient of health. In other words, a reef with large fish biomass does not necessarily have large coral cover. For instance, the site with the largest fish biomass in the Caribbean, the marine reserve of Jardines de la Reina, Cuba, has an average of 10% coral cover, similar to that in many other degraded reefs where fish biomass is much lower (Newman et al. 2006). Therefore, we need to use indicators for both reef fishes and benthic communities. While some previous studies have quantified the abundance and/or biomass of all reef fish species (as we did in our study), many other studies focused on selected groups of reef fishes, used sampling areas smaller than those where top predators are likely to be encountered, and only quantified fish numbers but not accurate fish sizes. We recommend other groups to quantify the abundance, size structure and biomass of all reef fish species that are surveyable using standard strip visual transects on SCUBA (see Deliverable #2). Abundance data and data on selected reef fish groups are not useful for comparison across reefs and regions within the scope of MMA. Many previous studies have also quantified coral cover alone, without paying attention to coral identification, coral size, and other substrata. We recommend the quantification of all major benthic substrata, the identification of corals to at least genus, and when possible the quantification of coral size structure.

Microbes are not the strongest descriptor at the community level, despite their marked monotonic shift across the gradient of human disturbance. However, microbial abundance is associated with the incidence of coral disease, and thus represents a strong indicator of the potential for future coral decline. We thus recommend quantification of the abundance of bacteria and Archaea to provide an indicator of the sensitivity of the reef to disease.

To test the indicators and to begin developing a ranking of global coral reef health, we have compiled some of the available data on fish biomass and coral cover (the two most readily available data in the literature; microbial data are very scarce) from different coral regions worldwide, and plotted them in a phase space (Fig. 7). We assumed that Kingman was the most pristine reef (100% pristine) and calculated the euclidean distance between the origin and Kingman; we then calculated the distance between each reef and the origin, and divided each by

the Kingman distance to the origin, to thus obtain the proportion of “pristinness” of each reef (Fig. 8). Please note that our analysis for Jamaica yields the same position along the gradient of degradation as in Pandolfi et al. (2003). The healthiest reefs are protected in no-take marine reserves (Fig. 8). In the next months we aim at completing the list of reef sites and performing a global reef health analysis.

## **Deliverable 2: Protocols for quantifying the set of indicators of coral reef health**

### *Reef fishes*

Surveys are conducted by one or two (preferable) teams of paired divers, with divers rotating between teams to distribute individual biases. Stations should be haphazardly placed at similar distances (in our Line Islands study we surveyed 25 sites per atoll, located approximately one kilometer apart) and provide a good spatial representation of each reef/atoll. Note that for consistency, sites should be sampled within similar habitat types. If multiple habitat types are under investigation, sufficient replication of stations should be repeated for each habitat.

At each station, one team of tandem-paired divers tally all fishes as they are encountered within fixed-length (25-m) strip transects whose widths differed depending on the size of fish enumerated. Transect bearings are determined haphazardly along isobaths ( $\pm 1$  m) (in our Line Islands study we focused on semi-exposed fore reef habitats between 10 and 12 meters depth). Each diver is responsible for one-half of the areas surveyed, as follows: large-bodied vagile fishes  $\geq 20$  cm total length (TL) are tallied within an 8-m wide strip (two, 4-m wide swaths separated by 1 m) surveyed on an initial “swim-out” as the transect line is laid. Divers look ahead and count only the sharks that are within the projected transect. Since sharks may be attracted by divers, divers should avoid counting sharks that cross the transects after an initial snapshot assessment to prevent overestimating their abundance. Small-bodied fish ( $< 20$  cm TL), which are generally less vagile and more site-attached than large-bodied fish, are tallied within a 4-m wide strip (two, abutting 2-m wide swaths centered on the transect line) surveyed on the return swim back along the laid line. Fishes are recorded by species or lowest recognizable taxon. Tallies are binned by 5-cm TL class. Three transects, each separated by about 10-m distance from its neighbor, should be surveyed at each station. Thus, at each station, the densities of large-bodied fishes are estimated within a  $600 \text{ m}^2$  ( $3 \times 25 \text{ m}$  by  $8 \text{ m}$ ) area, and the densities of small fishes within a  $300 \text{ m}^2$  ( $3 \times 25 \text{ m}$  by  $4 \text{ m}$ ) area.

Additional species richness and size frequency data should be recorded to complement those recorded on transects. Species presence are tallied within  $\sim 3,000 \text{ m}^2$  (100-m long by 30-m wide) areas searched by 1-way zigzag swims centered on the transect lines. All data are recorded on pre-printed forms bearing abbreviated names of the most abundant and commonly encountered species.

Transects provide the input to estimates of species- and size-specific numerical densities. Various published, unpublished, and web-based ([www.FishBase.org](http://www.FishBase.org)) sources provide length-weight regression parameters necessary for converting numbers to wet weight biomass. Densities are standardized to one hectare ( $\text{ha} = 10,000 \text{ m}^2$ ).

### *Benthic communities*

Quantitative assessments of the benthos are made using the photoquadrat method (Preskitt et al. 2004). At each site two 25 m transects are placed on the benthos parallel to shore, 25 m apart and at a constant depth. Ten points are randomly selected and surveyed per transect. At each point a photograph is taken using a digital camera connected to a tripod (1 m high) and a

frame (photographs covering an area of 0.5 m<sup>2</sup>). During surveys notes should be made for each quadrat and collections made for organisms that are unidentifiable in the field. Upon return to shore all photographs are edited using Adobe Photoshop v 7.0. Image analysis is completed using the program Photogrid 1.0. For each photograph 100 points are randomly generated and the organism under each point is identified. A total of at least 10 benthic sites should be surveyed at each island/atoll. All organisms are identified to the finest level of resolution possible (genus level for hard and soft corals, functional group for algal turfs and crustose coralline algae, and species level for macroalgae and macroinvertebrates when possible). In addition, coral density and size is measured in two 25m x 2m transects (100m<sup>2</sup> total area) per site; the identity (at least to genus level) and size of all corals with colony center within 1m of the transect is counted.

### *Microbial communities*

Marine microbial communities determine the biotic conditions under which reef organisms persist, and in particular, determine the level of stress that individual macro-organisms must endure. Two simple proxies of microbial activity, and thus potential stress to macro-organisms, are microbial counts and estimates of *Vibrio* concentrations.

Microbial counts provide a baseline concentration of bacteria, viruses, and protists in the reef's waters. Increased microbial concentrations are correlated with increased levels of potential pathogenic microbes and increased coral stress (and disease). Microbial counts are performed in the laboratory with any of a suite of approaches, from fluorescence microscopy to automated counting. Field sampling involves bulk water sample collection followed by filtration through fine filters (e.g., 0.02µm glass fiber filters). The filters are stored frozen (i.e., at least at -4°C) until laboratory analysis.

*Vibrio* counts provide an estimate of the levels of potential pathogens in the microbial community. Various techniques are available for collecting these data, and the specific field collection protocols vary with each technique. In general, density of culturable *Vibrio* is estimated by plating and incubating pre-filtered water on culture plates. Note that *Vibrio* strains include many potential pathogens, and all handling and disposal techniques require focus on personal health and safety.

Although standard methodologies are available for each analysis, data collection involves use of a microbiological laboratory. We recommend collaboration with a suitable colleague to accomplish these analyses. Because of the many variables involved in laboratory analyses and lab-to-lab variation in techniques, the precise details of each type of field collection should be worked out with the laboratory-based colleague prior to commencement of the work.

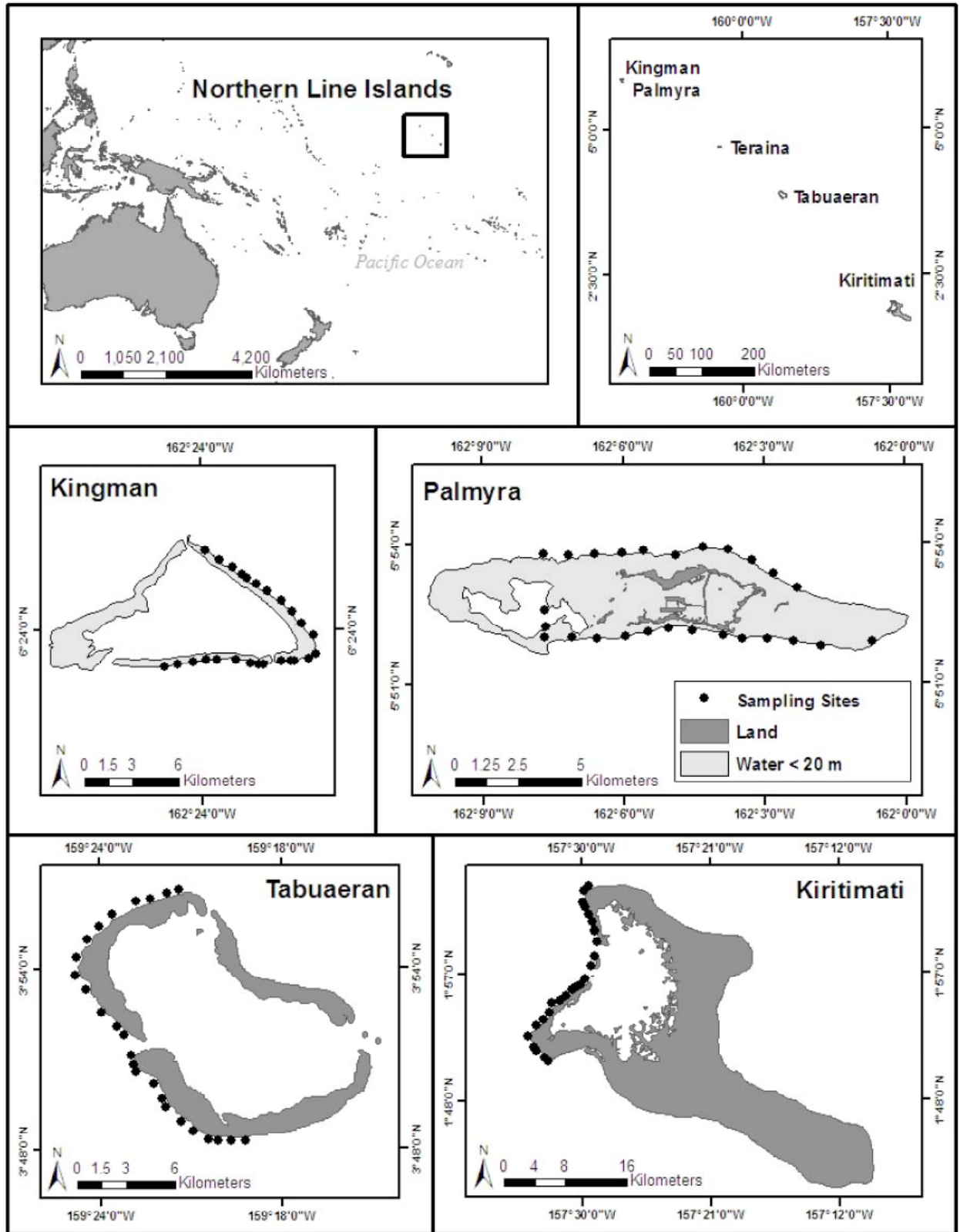
## **SCIENCE TO ACTION**

We have worked with media worldwide to communicate the results of our study. After publication of two papers in PLoS ONE and one essay in PLoS Biology in February 2008, our results were featured in a number of media, including The New York Times, The Washington Post, the San Diego Union Tribune, El País, La Vanguardia, and several other newspapers and radio stations in the US and Spain.

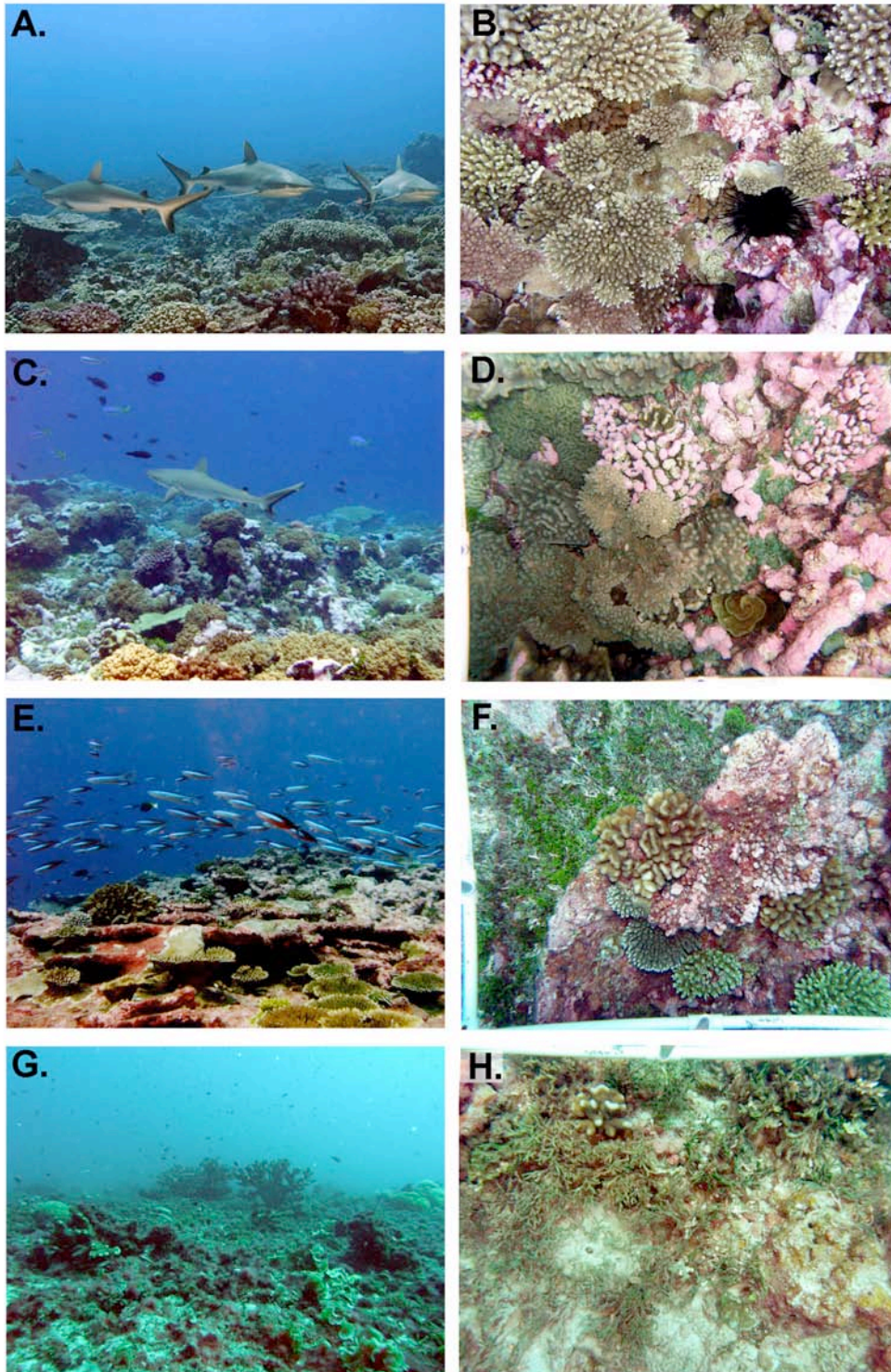
In addition to the scientific papers (attached), we have presented the results at the European Coral Reef Conference in 2007, and several communications will be presented at the International Coral Reef Symposium in Fort Lauderdale in July 2008.

We made continuous attempts to communicate directly with Kiribati government officials

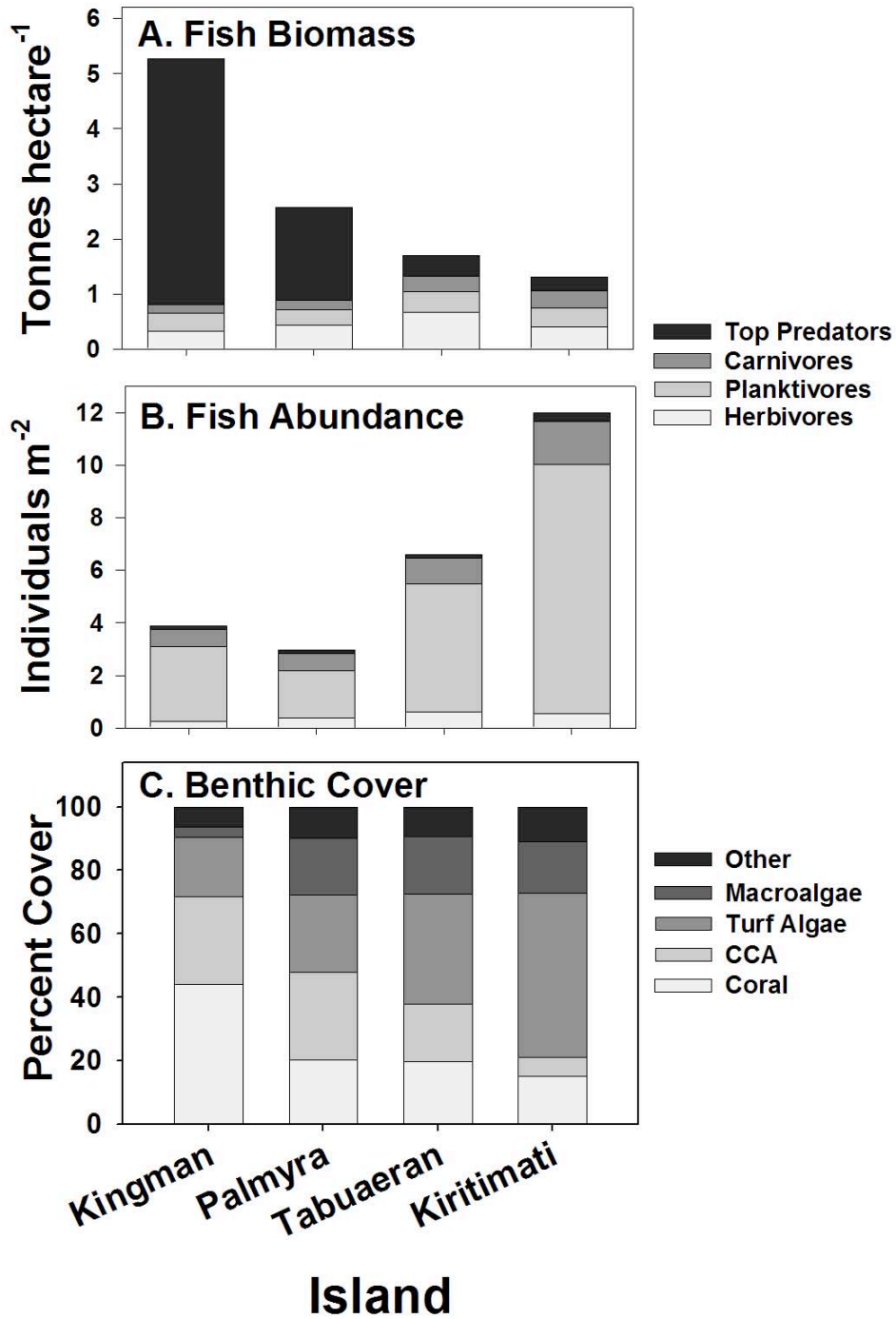
about the results and the implications of our study, but we have not been successful. Sue Taei of Conservation International and Greg Stone of the New England Aquarium have helped us with introductions and giving our reports to government officials. In 2008 Sala expects to travel to Tarawa to communicate directly with them.



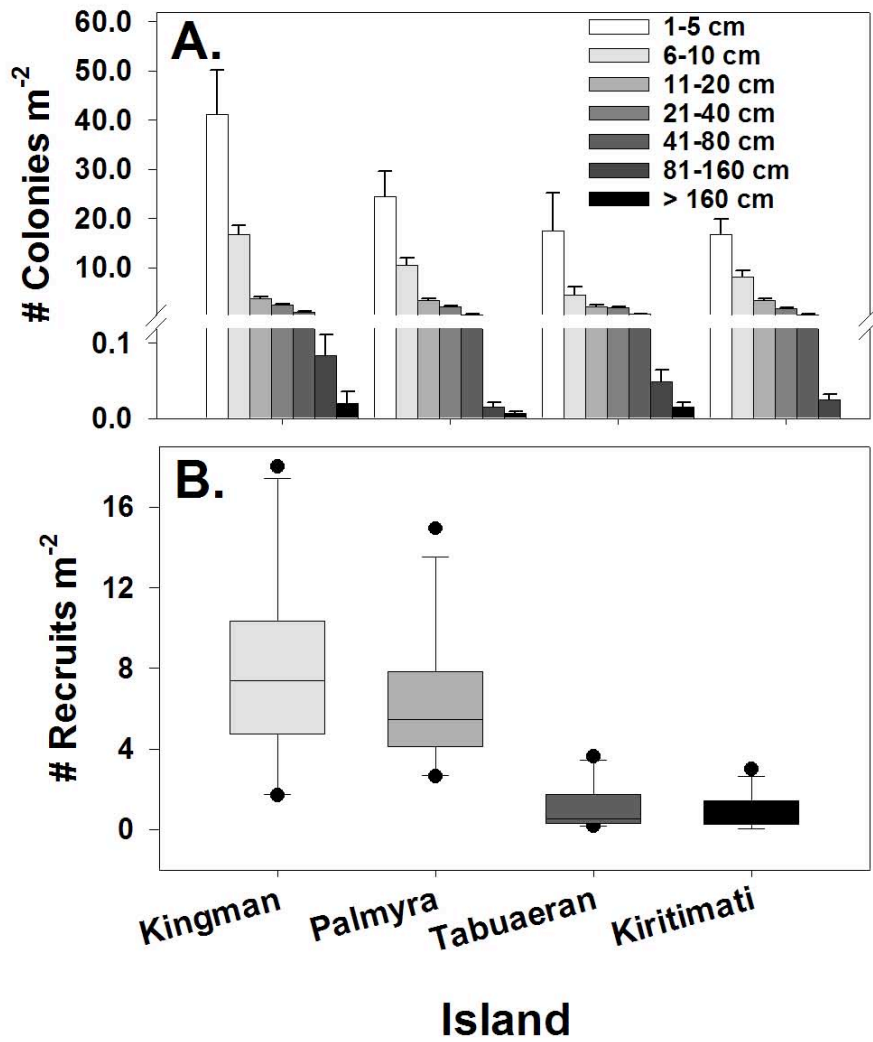
**Figure 1.** Location of study sites on the northern Line Islands.



**Figure 2.** General aspect of fore reef habitats (left column) and representative 1-m<sup>2</sup> photos of the bottom (right column) at Kingman (A-B), Palmyra (C-D), Tabuaeran (E-F), and Kiritimati (G-H). Photo credits: A by Zafer Kizilkaya, B-H by Jennifer Smith.



**Figure 3.** Fish abundance (A) and biomass (B), and cover of major benthic functional groups at Kingman, Palmyra, Tabuaeran and Kiritimati. CCA = crustose coralline algae.



**Figure 4.** A. Size frequency distribution of corals. B. Coral recruitment as determined by the number of colonies of *Acropora*, *Pocillopora* and Fungiids less than 5 cm in diameter per  $\text{m}^{-2}$ .

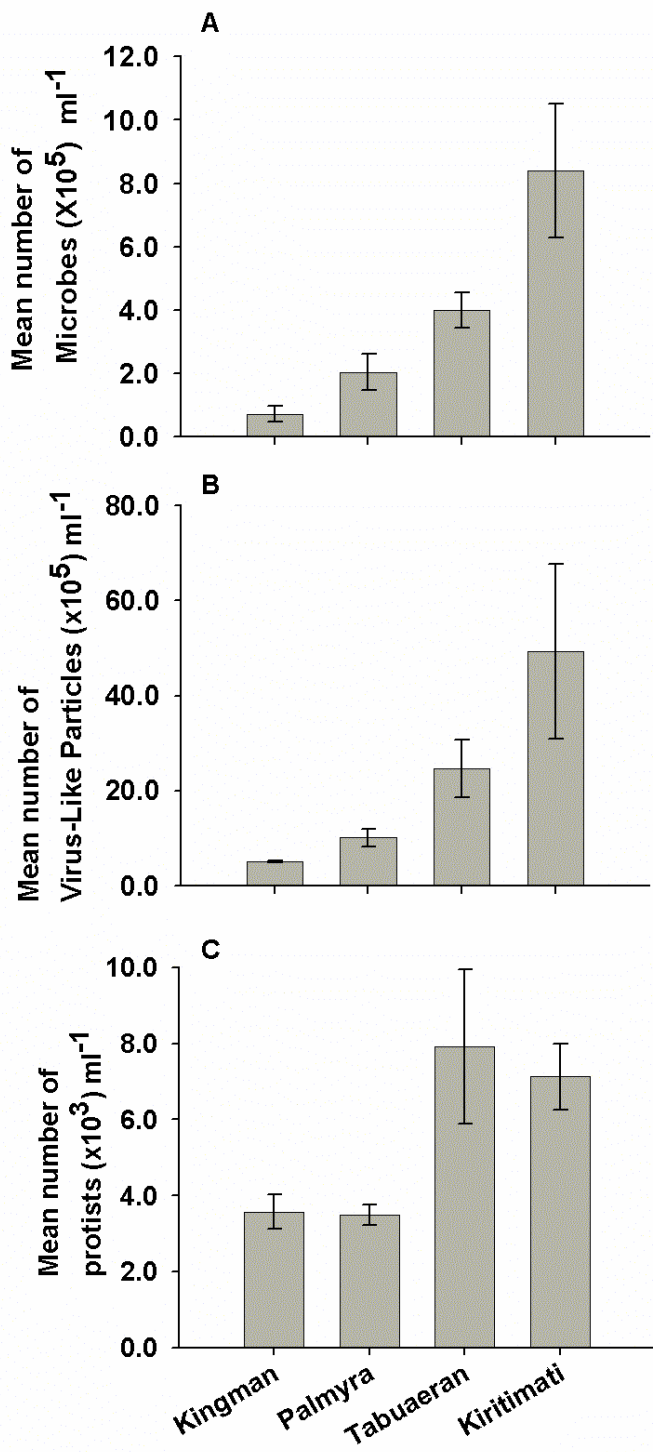
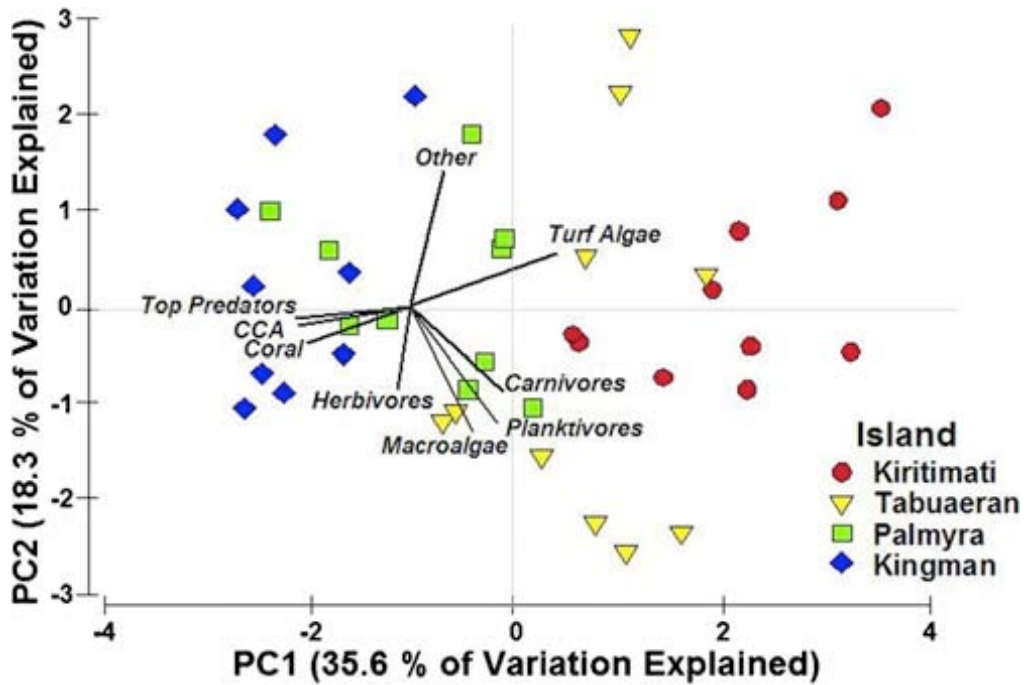
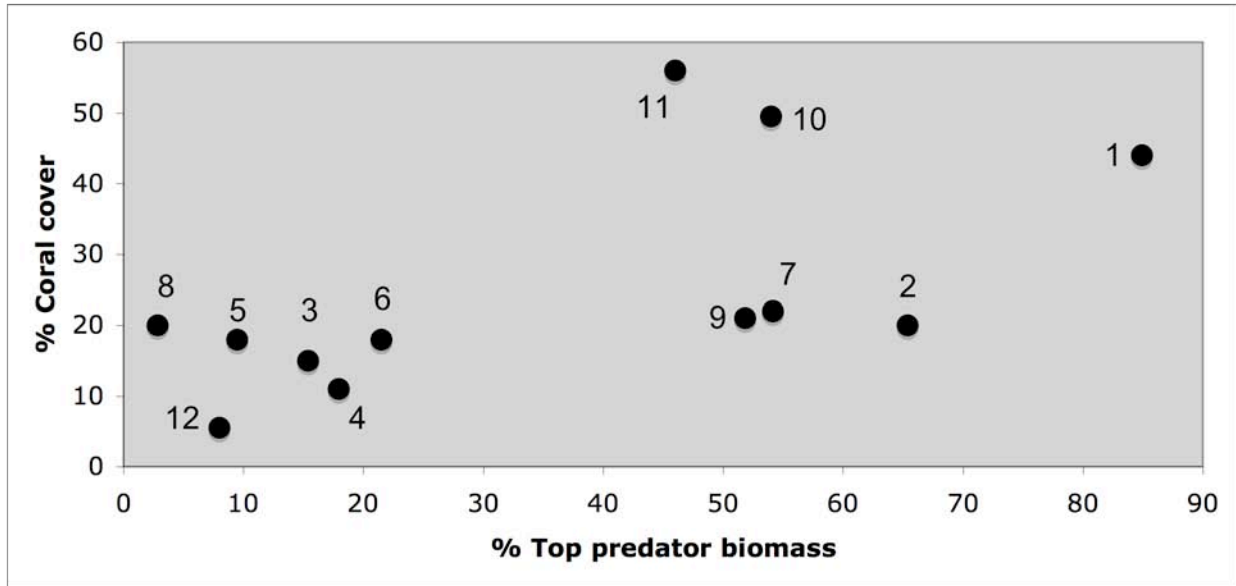


Figure 1

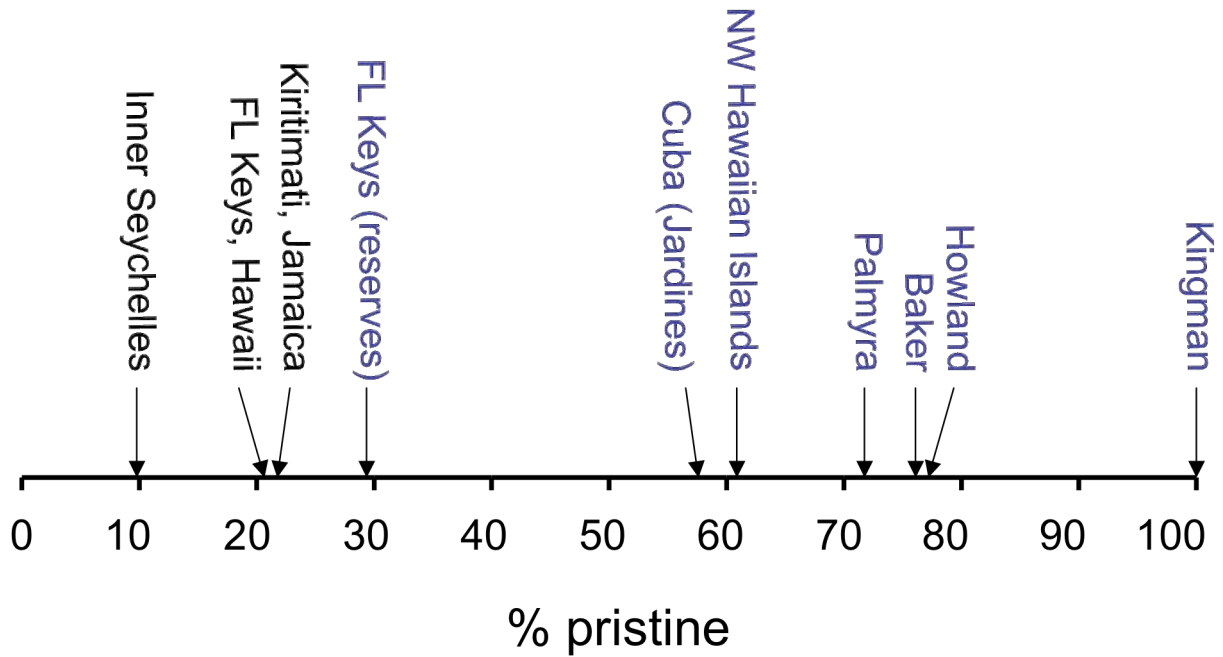
**Figure 5.** A. Abundance of microbes, virus-like particles, and protists across the northern Line Islands.



**Figure 6.** Principal component analysis (PCA) of major fish and benthic groups from all sites with photoquadrat data ( $n = 10$  per atoll, except Kingman with  $n = 9$ ). Atolls cluster in sequence along the first principal component axis (PC1), shifting from left to right from Kingman to Kiritimati. The major loadings on PC1 include biomass of top predatory fish, coral and CCA cover (to left, i.e., decreasing disturbance) and turf algal cover (to right, i.e., increasing disturbance). Note that herbivore biomass, the putative intermediate in models of simple trophic cascades, is orthogonal to the dominant axis correlated with apex predators..



**Figure 7.** Relationship between the health of the fish assemblage (as proportion of the total fish biomass accounted for by top predators) and the health of the benthic community (as the percent cover of live coral) for 12 coral reef regions. Pristine reefs are located near the upper right corner of the graph. 1: Kingman, 2: Palmyra, 3: Kiritimati, 4: Jamaica, 5: Florida Keys (unprotected), 6: Florida Keys (no-take marine reserves), 7: NW Hawaiian Islands, 8: Main Hawaiian Islands, 9: Cuba (Jardines de la Reina marine reserve), 10: Howland, 11: Baker, 12: Inner Seychelles



**Figure 8.** Ranking of 12 coral reefs along a gradient of health/degradation. For the purpose of this exercise we assume that Kingman Reef is 100% pristine (that is, as pristine as we know). Names in blue are no-take marine reserves.

# Baselines and Degradation of Coral Reefs in the Northern Line Islands

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## Abstract

Effective conservation requires rigorous baselines of pristine conditions to assess the impacts of human activities and to evaluate the efficacy of management. Most coral reefs are moderately to severely degraded by local human activities such as fishing and pollution as well as global change, hence it is difficult to separate local from global effects. To this end, we surveyed coral reefs on uninhabited atolls in the northern Line Islands to provide a baseline of reef community structure, and on increasingly populated atolls to document changes associated with human activities. We found that top predators and reef-building organisms dominated unpopulated Kingman and Palmyra, while small planktivorous fishes and fleshy algae dominated the populated atolls of Tabuaeran and Kiritimati. Sharks and other top predators overwhelmed the fish assemblages on Kingman and Palmyra so that the biomass pyramid was inverted (top-heavy). In contrast, the biomass pyramid at Tabuaeran and Kiritimati exhibited the typical bottom-heavy pattern. Reefs without people exhibited less coral disease and greater coral recruitment relative to more inhabited reefs. Thus, protection from overfishing and pollution appears to increase the resilience of reef ecosystems to the effects of global warming.

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## Introduction

Early historical accounts of coral reefs describe an abundance of sharks and other large fishes and luxuriant coral growth that seem incredible in the context of today's coral reefs and modern reef science [1–6]. Quantitative surveys conducted as recently as the 1980s report surprisingly high densities of predatory fishes (e.g., sharks and groupers) and corals; [7–9]. In contrast, most recent accounts of reefs largely describe dramatic declines of entire guilds of large fishes and corals [10–15]. The decline of large predators is believed to affect strongly patterns of trophic flow in marine communities [16,17], and declines in coral cover have been linked to decreases in abundance and diversity of reef fishes [18,19]. Nevertheless, we still lack a comprehensive, quantitative description of the structure and functioning of pristine coral reef communities.

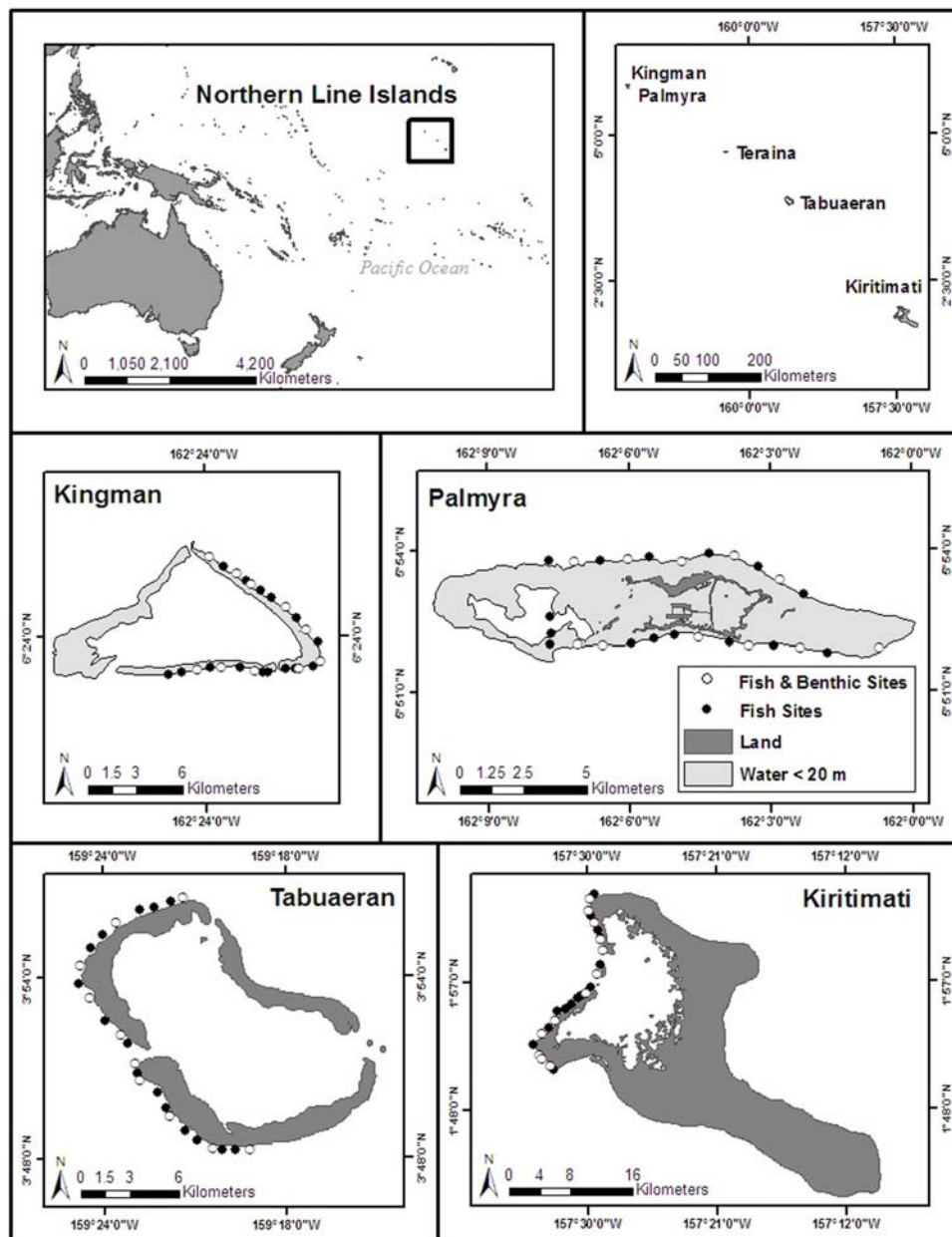
Ecological baselines of the structure and functioning of ecosystems in the absence of human impacts can provide fundamental insights for conservation and restoration [20]. For example, historical reconstructions of the frequency of fires in temperate forests have revealed the unanticipated ecological consequences of modern fire suppression [21]. Knowledge of baseline fire conditions and the positive ecosystem services afforded by occasional fires have played an important role in forest restoration and management [22]. Protected areas also can be used as ecological reference sites [20,23,24]. For example, studies of wolves in Yellowstone National Park (a large, protected area) have helped to quantify the ecological roles filled by these top predators. Wolves provide a consistent source of carrion (from incompletely consumed prey carcasses) for scavenger species, dampening the increasing fluctuations of food availability caused by climate change [25]. Thus, insights into the baseline structure

and functioning of ecosystems are critical for development of effective conservation and restoration programs [26].

Recent surveys of the fishes of uninhabited, remote coral reefs in the Northwestern Hawaiian Islands and the northern Line Islands [23,27] strongly support historical reports of great fish abundance and predator domination that characterized coral reefs before extensive fishing efforts (see historical references for the Line Islands in Table S2). Comparisons of these data with neighboring, inhabited sites suggest that these coral reef assemblages have been altered by human activities, mostly fishing [23,27]. However, most reef studies focus on individual taxa and do not provide community-wide descriptions of baseline conditions. With very few exceptions, studies of reef communities across gradients of human disturbance have

been conducted on reefs that were previously severely degraded and are only now recovering to varying degrees due to deliberate protection from fishing [12,28–30].

Palmyra and Kingman atolls in the northern Line Islands provide the opportunity to study reefs that have experienced minimal human impacts compared to most other reefs. Fishing pressure and oceanographic stress from upwelling or sea surface temperature anomalies are low in comparison with neighboring Tabuaeran and Kiritimati in the same biogeographic region (Fig. 1; also see Table 1 and Supplemental Data S1 for a detailed summary of anthropogenic and oceanographic conditions in the region). We surveyed in detail the fishes, corals, macroinvertebrates, and macroalgal assemblages across the four atolls to



**Figure 1. Location of study sites at Kingman, Palmyra, Tabuaeran and Kiritimati atolls in the Line Islands.** Sites were located at semi-exposed fore reef habitats between 10 and 12 m in depth, and approximately one km apart from each other. Reef fishes were surveyed at all sites ( $n = 25$  sites per atoll), and benthic communities at a subset of sites ( $n = 10$ – $12$  sites per atoll). doi:10.1371/journal.pone.0001548.g001

**Table 1.** Characteristics of the seven central Pacific atolls, including the four northern Line Islands and three additional equatorial atolls.

	Human involvement						Environment					
	Perimeter (km at 10 m isobath)	Population (2005 est.)	Pop. density (#/inhabited km reef <sup>-1</sup> )	Fishing activities	Agricultural activities	Other activities	SST (°C)	Mean max. annual # of DHW	Number of fish species	Coral cover (%)	Number of coral species	
<b>Kingman</b>	34.4	0	0	Prohibited	-	-	27.9 (0.7)	1.32 (1.61)	-	-	-	
<b>Palmyra</b>	40.8	20	0.5	Prohibited	Copra (historic)	Navy base (1940–46)	27.9 (0.8)	2.69 (3.76)	-	-	-	
<b>Tabuaeran</b>	52.3	2,539	62.2	Subsistence	Seaweed, copra	Shipwrecks	27.5 (0.9)	3.32 (4.45)	-	-	-	
<b>Kiritimati</b>	146.8	5,115	108.9	Subsistence/commercial	Seaweed, copra	Nuclear tests, shipwrecks	27.1 (1.1)	5.47 (6.48)	-	-	-	
<b>Jarvis</b>	12.4	0	0	Prohibited	Guano removal	Small habitation (early 1900s)	27.0 (1.2)	6.99 (8.14)	-	-	-	
<b>Howland</b>	8.9	0	0	Prohibited	Guano removal	Limited military (1930s–40s)	28.1 (1.0)	6.80 (6.49)	-	-	-	
<b>Baker</b>	9.7	0	0	Prohibited	Guano removal	Limited military (1930s–40s)	28.1 (1.0)	6.82 (6.43)	-	-	-	
Environment (cont.)												
Annual freq. of NOAA Level 2 bleaching events	Recent years of Level 2 events	Chl <i>a</i> (mg m <sup>-3</sup> )	<i>In situ</i> Chl <i>a</i> (mg m <sup>-3</sup> )	DIN (μM L <sup>-1</sup> )	SRP (μM L <sup>-1</sup> )	Biomass of total fishes (mt ha <sup>-1</sup> ; w/percent piscivorous in brackets)	Number of fish species	Coral cover (%)	Number of coral species	Biota		
										Chl <i>a</i> (mg m <sup>-3</sup> )	<i>In situ</i> Chl <i>a</i> (mg m <sup>-3</sup> )	
0%	-	0.10 (0.05)	0.046±0.005	1.00±0.14	0.107±0.007	5.3±0.7 [85%] <sup>b,c</sup>	232 [223, 244]	43.8±5.4 <sup>b,c</sup>	106 [101, 114]	-	-	
14%	1994, 2002	0.10 (0.05)	0.069±0.013	1.75±0.44	0.120±0.012	2.6±0.3 [65%] <sup>b,c</sup>	234 [227, 244]	20.4±2.7 <sup>b,c</sup>	102 [93, 113]	-	-	
19%	1994, 1997, 2002	0.15 (0.03)	0.146±0.030	2.51±0.25	0.197±0.016	1.7±0.2 [24%] <sup>b</sup>	250 [243, 260]	19.5±4.0 <sup>b</sup>	103 [97, 111]	-	-	
19%	1991, 1997, 2002	0.16 (0.02)	0.247±0.042	3.61±0.19	0.295±0.036	1.3±0.1 [15%] <sup>b</sup>	281 [273, 292]	15.0±2.3 <sup>b</sup>	83 [78, 90]	-	-	
33%	1991, 1992, 1994, 1997, 2002	0.18 (0.02)	N/A	N/A	N/A	8.0±2.1 [74%] <sup>a</sup>	-	43.9±8.0 <sup>a</sup>	-	-	-	
38%	1992, 1993, 1994, 2002, 2004	0.16 (0.05)	N/A	N/A	N/A	4.2±0.2 [54%] <sup>a</sup>	-	49.5±5.9 <sup>a</sup>	-	-	-	
38%	1992, 1993, 1994, 2002, 2004	0.16 (0.05)	N/A	N/A	N/A	3.9±0.2 [46%] <sup>a</sup>	-	55.6±5.1 <sup>a</sup>	-	-	-	

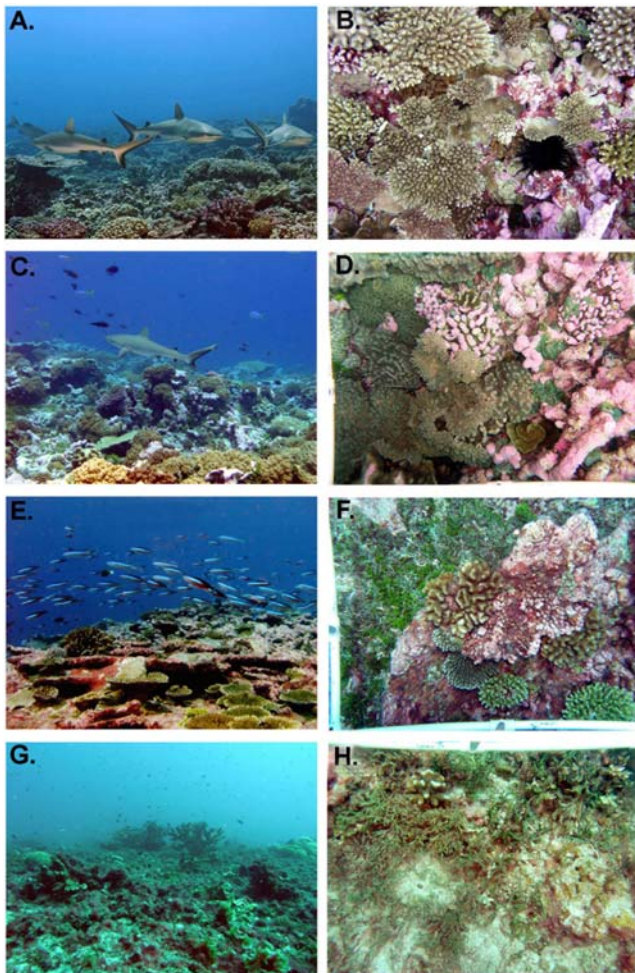
Quantitative environment and biota data are presented as means with variability presented as either standard error (reported with '±'), standard deviation (reported in parentheses), or 95% confidence intervals (reported in brackets). Abbreviations are as follows: SST, Sea surface temperature; DHW, Degree heating weeks; Chl *a*, Chlorophyll *a*; DIN, Dissolved inorganic nitrogen; SRP, Soluble reactive phosphorus. Details on the estimations and calculations are presented in the Methods and Supplemental Data.

<sup>a</sup>Unpublished data from NOAA Fisheries, Pacific Islands Fisheries Science Center, Coral Reef Ecosystem Division and US Fish and Wildlife Service. These data were collected using similar methods as those used in this study.

<sup>b</sup>This study

<sup>c</sup>NOAA data showed a total fish biomass of 1020 g m<sup>-2</sup> and 49% coral cover at Kingman, and 520 g m<sup>-2</sup> and 45% at Palmyra. Differences in estimates of biomass across the different data sets may be due to a difference in sampling effort, higher variability in habitat types in the NOAA data, and changes over time.

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**Figure 2. General aspect of fore reef habitats (left column) and representative 0.5-m<sup>2</sup> photos of the bottom (right column) at Kingman (A–B), Palmyra (C–D), Tabuaeran (E–F), and Kiritimati (G–H), showing the degradation from a reef dominated by top predators and corals (Kingman) to a reef dominated by small planktivorous fishes and algae. Photo credits: A by Zafer Kizilkaya, B–H by Jennifer Smith.**  
doi:10.1371/journal.pone.0001548.g002

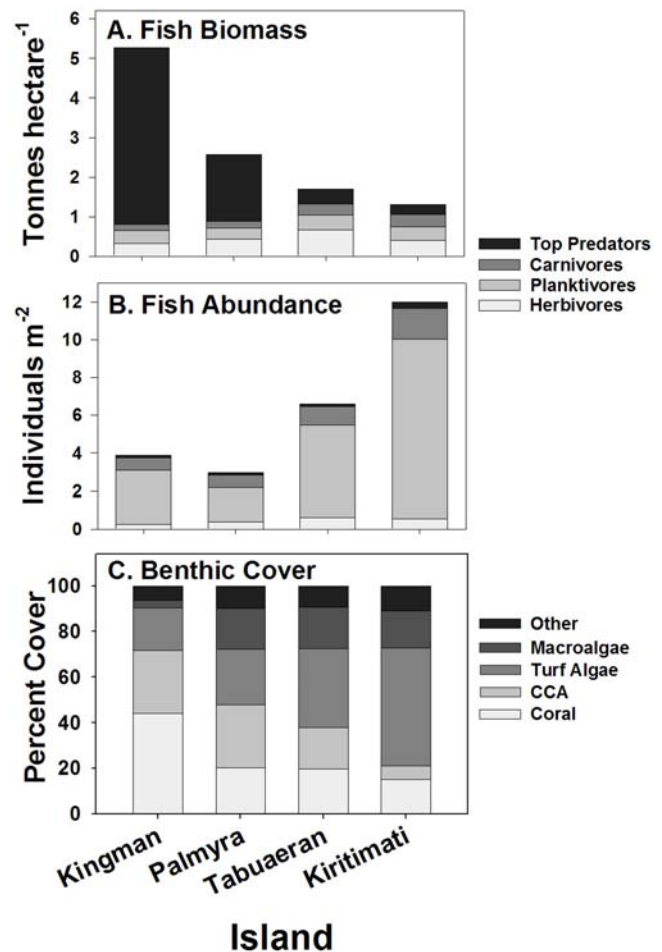
evaluate the effects of increasing human populations at Kiritimati and Tabuaeran relative to the baselines of Palmyra and Kingman. Detailed description of the microbial community is reported in a companion paper [31].

## Results

### Reef Fishes

Biomass and abundance of reef fish varied greatly from Kingman to Kiritimati (Fig. 2). Total fish biomass decreased from 527 to 132 g m<sup>-2</sup> (1-way ANOVA on atoll effect,  $F_{3,97} = 27.6$ ,  $p < 0.0001$ ; Fig. 3A) whereas total abundance increased from 4 to 12 fishes m<sup>-2</sup> ( $F_{3,97} = 69.2$ ,  $p < 0.0001$ ; Fig. 3B). This contrasting pattern of fish biomass and abundance reflects a shift from dominance by a few large top predators at Kingman to many much smaller, lower trophic level consumers, especially planktivores, at Kiritimati. Species richness increased from Kingman to Kiritimati, generally tracking the increase in fish abundance (Table 1, Fig. 3B).

Top predators accounted for 85% of total fish biomass at Kingman, and decreased to 19% at Kiritimati (Fig. 3A). The



**Figure 3. Fish biomass (A) and abundance (B), and cover of major benthic functional groups (C) across the northern Line Islands.** Note that human impacts increase from left to right in this and subsequent figures. CCA = crustose coralline algae.  
doi:10.1371/journal.pone.0001548.g003

dominant predators at Kingman were snappers, jacks and sharks (median total length = 33 cm, maximum = 200 cm) versus small groupers (principally Epinephelinae; median = 13 cm, maximum = 65 cm) at Kiritimati. Sharks comprised 74% of the top predator biomass (329 g m<sup>-2</sup>) at Kingman and 57% at Palmyra (97 g m<sup>-2</sup>), whereas they were virtually absent at Tabuaeran and Kiritimati. Thus, the typical fish biomass pyramid observed at most reefs around the world today [12,32,33], including those of Tabuaeran and Kiritimati, is inverted at Kingman and Palmyra. Inverted biomass pyramids of fishes have only been documented elsewhere in the Northwestern Hawaiian Islands [23]. Thermodynamic constraints, however, require that inverted biomass pyramids be supported by bottom-heavy pyramids of production [34,35]. This suggests that turnover rates of predators are much lower than of their prey, and that trophic efficiency is high at all levels. Such dramatic alterations of rates and pathways of trophic flow have been afforded little research effort to date (see notable exception in [36]).

The structure of lower trophic level fish assemblages also changed across the gradient. Carnivores (principally predators upon invertebrates) had lower biomass at Kingman and Palmyra than at Tabuaeran and Kiritimati ( $F_{3,97} = 68.1$ ,  $p < 0.0001$ ). Small planktivores only a few centimeters in length were the most numerous fish at all atolls, especially at Kiritimati, but planktivores

accounted for only 6% of total fish biomass at Kingman compared to 26% at Kiritimati (Fig. 3). The biomass of herbivorous fishes was greatest at Tabuaeran ( $F_{3,97}=8.4$ ,  $p<0.0001$ ) but did not differ among the other atolls. The mean per capita mass of herbivores was smallest at Kiritimati ( $F_{3,97}=8.9$ ,  $p<0.0001$ ), driven largely by a 60-fold increase in biomass of small-bodied, territorial damselfish from Kingman to Kiritimati.

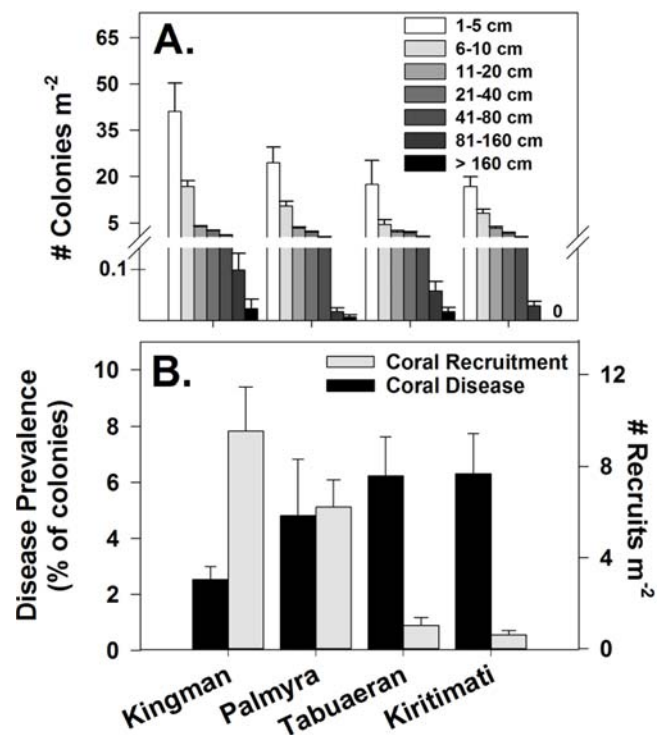
The changes in reef fish assemblage structure are best described as a response to increased fishing pressure from Kingman to Kiritimati (Supplemental Data). Fishing pressure tends to disproportionately reduce densities of longer-lived, larger-bodied individuals [37,38], which are frequently from higher trophic levels [39]. Recent studies indicate that the loss of live coral cover, for example due to bleaching, can reduce the density of small-bodied fishes that seek food and/or shelter in, or recruit to, the living coral matrix [11,18]. In the northern Line Islands, in contrast, decreasing coral cover was associated with an increase in the abundance of smaller fishes (Fig. 3), suggesting that the effects of coral loss on fish assemblage structure are secondary to (although likely reinforce) fishing impacts.

### Benthic Community

Benthic community structure shifted from domination by reef-building stony corals and crustose coralline algae (CCA) at Kingman and Palmyra to domination by macroalgae (including species of *Halimeda*, *Caulerpa*, *Avrainvillea*, *Dictyosphaeria*, and *Lobophora*) and algal turfs at Tabuaeran and especially Kiritimati (PERMANOVA,  $F=14.1$ ,  $p=0.001$ ) (Figs. 2 and 3C). Stony corals plus CCA strongly dominated the reefs at Kingman (71% cover; Fig. 3C) with numerous large coral colonies (primarily plate-forming and branching *Acropora* spp.). Cover of stony corals plus CCA dropped to 48% at Palmyra, 38% at Tabuaeran, and 21% at Kiritimati (Fig. 3C). Benthic community composition was much more variable at Tabuaeran than the other atolls, ranging from sites dominated by CCA and *Acropora* corals (maximum 63% combined cover) to sites dominated by fleshy algae (79% combined cover of turf and macro-algae). However, turf algae were the most common benthic group overall (36% cover; Fig. 3C), whereas dead corals carpeted by turf and macroalgae more uniformly dominated the reefs at Kiritimati (68% mean combined cover).

Coral density (numbers of colonies  $m^{-2}$ ) tracked coral cover and was highest at Kingman and lowest at Kiritimati (ANOVA,  $F_{3,304}=3.1$ ,  $p<0.03$ ) (Fig. 4A). However, the size frequency distributions of corals were statistically indistinguishable among atolls (size class  $\times$  atoll interaction,  $F_{3,300}=1.8$ ,  $p>0.1$ ). Nevertheless, we found no colonies greater than 160 cm at Kiritimati, although there were numerous smaller colonies apparently resulting from the partial mortality of previously larger diseased or degenerating colonies. Coral species richness across the sampled area decreased from Kingman to Kiritimati, paralleling the decrease in density (Table 1).

We surveyed 24 species of mobile macroinvertebrates and 21 morphospecies of sessile (non reef-building) macroinvertebrates. In general, exposed and conspicuous macroinvertebrates were uncommon at all atolls with no obvious correlation with human abundance (Table S3). Sea urchins were more abundant at Kingman (24.6 individuals  $100 m^{-2}$ ) than at any of the other atolls (range = 0–8.2  $100 m^{-2}$ ; non-parametric median test,  $p<0.0001$ ) primarily due to *Echinothrix diadema* ( $p<0.0001$ ) (Tables S3 and S4). Holothurians (1.1 individuals  $100 m^{-2}$ ;  $p=0.03$ ) and giant clams (*Tridacna maxima*; 0.75 individuals  $100 m^{-2}$ ;  $p=0.018$ ) were also more abundant at Kingman than elsewhere. These animals are targeted for consumptive harvest at Kiritimati (Tables S3 and S4).



**Figure 4. Size frequency distribution of corals (A), and coral disease prevalence and recruit density (B) across the northern Line Islands.** Because recession in adult corals can often lead to fragmentation and the development of many small colonies that may have all been part of one larger colony, recruits are defined here as colonies less than 5 cm in diameter from coral taxa demonstrating essentially unidirectional growth (i.e., *Acropora*, *Pocillopora* and *Fungiids*), and thus represent individuals known to be young relative to larger individuals. Note that there were no colonies larger than 160 cm at Kiritimati.

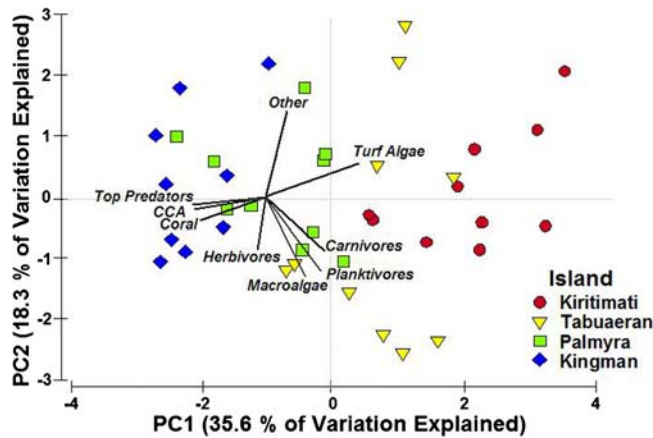
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### Coral Recruitment and Disease

Numbers of small colonies (1–5 cm) of *Acropora*, *Pocillopora* and *Fungiids*, taxa that could be definitively identified as recruits, were more than 6 times higher at Kingman and Palmyra than at Tabuaeran and Kiritimati (Fig. 4; ANOVA,  $F_{3,40}=25.9$ ,  $p<0.001$ ; Fig. 4A). These differences were robust after correcting for inter-atoll differences in total coral density (i.e., densities of recruits standardized by the mean atoll-specific total densities of colonies  $<5$  cm;  $F_{3,40}=15.8$ ,  $p<0.001$ ). Prevalence of coral disease showed the opposite pattern, and was lowest at Kingman and highest at Kiritimati and Tabuaeran (Kruskal Wallis test;  $H=8.0$ ,  $df=3$ ,  $p=0.04$ ) (Fig. 4B). Notably, the pattern of disease prevalence paralleled that of microbial densities both on the benthos and in the water column (see [31] for a more detailed treatment of these findings).

### Coral Reef Community Structure: a Food-Web Perspective

We used principal component analysis (PCA) to explore the correlations among density estimates for dominant functional groups (four fish guilds and five benthic types) for all sites at which both benthic photoquadrat and fish census data were collected (Fig. 5). The first two principal component axes (PC1 and PC2) described over 50% of the variation in the data and thus provide insights into the dominant correlations among these data [40]. PC1 paralleled the gradient of atolls, with sites from each atoll



**Figure 5. Principal component analysis (PCA) of major fish and benthic groups from all sites with photoquadrat data (n = 10 per atoll, except Kingman with n = 9).** Atolls cluster in sequence along the first principal component axis (PC1), shifting from left to right from Kingman to Kiritimati. The major loadings on PC1 include biomass of top predatory fish, coral and CCA cover (to left, i.e., decreasing disturbance) and turf algal cover (to right, i.e., increasing disturbance). Note that herbivore biomass, the putative intermediate in models of simple trophic cascades, is orthogonal to the dominant axis correlated with apex predators.  
doi:10.1371/journal.pone.0001548.g005

clustering together and organized in sequence (left to right from Kingman to Kiritimati, Fig. 5). The major loadings of PC1 were biomass of top-predatory fish, coral cover, and CCA cover in the direction of Kingman versus turf algal cover in the opposite direction. Loadings for biomass of non-predatory fishes were generally orthogonal to the loading for predator biomass. As such, there was no evidence of a simple trophic cascade within the reef food web, contrary to some theoretical predictions [41,42].

The mechanisms leading to the marked association between high coral cover, low cover of turf, and abundant top predators across sites (as noted along PC1) are unclear. In contrast to other observational studies [43], there was no obvious correlation between the abundance or biomass of herbivorous fishes and benthic community structure. Herbivore populations remained relatively constant (but generally high in comparison to other reefs [23]) across the atolls. Systematic shifts in the size-structure of herbivorous fish (smallest on Kiritimati) or in the relative abundance of functional types (increase of territorial damselfish from Kingman to Kiritimati) may have altered patterns of herbivory contributing to the observed differences in benthic structure. Further, grazing by invertebrates may also help to explain the differences in algal communities across atolls as sea urchins were most abundant on Kingman. An alternative explanation for the low macroalgal cover and high coral cover (and recruitment) at Kingman is that higher coral cover concentrates the grazing of herbivorous fish. Most herbivorous fish in the Pacific feed primarily on algae instead of corals [33], causing the intensity of herbivory on non-coral substrata to be greater at sites where coral cover is high [44]. However, if it is assumed that each atoll had comparable coral cover prior to human arrival and recent climate change, this mechanism of concentrating herbivory is inadequate to explain the original loss of live coral cover at Tabuaeran and Kiritimati.

### Diversity and Disturbance

Changes in species diversity were inconsistent across taxonomic groups: the number of coral species counted decreased from

Kingman to Kiritimati whereas the number of fish species increased (Table 1). Decreasing diversity with increasing human disturbance is commonly assumed, and has been reported at other sites in the past for both corals [45] and fishes [46,47]. In a recent study, Tittensor et al [47] report decreasing fish diversity from Palmyra to Kiritimati (they did not sample Kingman), which is in contrast to our findings. However, their analysis is not directly comparable because it was done at the family level and different habitats were analyzed on different atolls (back-reefs on Palmyra, fore-reefs on Kiritimati and Tabuaeran); they also surprisingly failed to observe significant differences in benthic composition among atolls, in contrast to the strong differences documented here (Fig. 3C).

Both intermediate disturbance models [48,49] and abundance-based models [50,51] are consistent with the diversity patterns we observed. A recent meta-analysis documented an increase in fish species richness with moderate declines in coral cover that shifted to a decrease in richness with more severe declines of coral [52], consistent with the model of intermediate disturbance. But regardless of the underlying cause, our results suggest that diversity alone is not a clear indicator of ecosystem health or condition, particularly across gradients that include undisturbed habitats like Kingman.

### Local Human Impacts and Oceanography across the Northern Line Islands

Kingman, Palmyra, Tabuaeran, and Kiritimati vary in human population size from uninhabited to moderately populated (Table 1). Unpopulated Kingman lacks permanent emergent land, is protected as a National Wildlife Refuge by the United States Fish and Wildlife Service (USFWS), and is not fished. Palmyra Atoll was physically altered by extensive dredging during the 1940s and was historically fished, but currently is fully protected from fishing by the USFWS. The camp at Palmyra has a maximum population of 20 people with modern sewage treatment. Tabuaeran has a permanent, growing population, estimated at 2,500 people in 2005, that subsists on fishing and has no sewage treatment. Kiritimati has an even more rapidly growing population, estimated to be 5,100 in 2005, that subsists primarily on fishing and lacks a functioning waste disposal system. Kiritimati also supports a commercial fishing operation for both food and aquarium fish (see the Supplemental Data S1 for further detail). Based on fisheries surveys, however, Tabuaeran and Kiritimati experience appreciable fishing with subsistence harvest yielding 20 t yr<sup>-1</sup> and 408 t yr<sup>-1</sup>, respectively, directly from the reef. Over 50% of the reef fishery is composed of predatory species. Comparing these estimates with our *in situ* counts (Figure 3), this yield is analogous to the complete removal annually of predatory fish from 27 and 800 ha of fore reef at Tabuaeran and Kiritimati, respectively.

We did not measure nutrient flow from human settlements to the reef at Tabuaeran and Kiritimati, but a simple calculation suggests that anthropogenic runoff is of minor importance. Industrial agriculture and manufacturing are absent and the primary input is sewage (Supplemental Data S1). Assuming that the average person excretes 10 g of nitrogen per day [53], the per capita annual nitrogen input to the watershed would be approximately 3.5 kg yr<sup>-1</sup>. This is <5% of the typical per capita impact of industrialized, non-insular populations [54], which suggests a weak effect of direct input by humans to the observed gradient in nutrient concentrations across the archipelago (Table 1).

The gradient in human habitation and fishing in the northern Line Islands parallels that of several oceanographic factors,

confounding interpretation of the observed patterns in the biota and human populations. Sea surface temperature (SST) generally increases with northern latitude in the region, although annual mean SST varies by just 0.8°C between the atolls (Table 1, Figure S1). The more equatorial atolls, however, experience greater year-to-year variability in SST, mainly due to El Niño Southern Oscillation (ENSO) events. Mean annual accumulation of degree heating weeks (DHW, see Methods for definition) increases approximately 4-fold from Kingman to Kiritimati (Table 1). Thus, reefs of Kingman have experienced significantly fewer large-scale bleaching episodes than those of Kiritimati (*sensu* [55–57]). There is also an increase in productivity from north to south associated with stronger upwelling [58,59]. Mean concentrations of total dissolved inorganic nitrogen (DIN), soluble reactive phosphorous (SRP), and chlorophyll *a* each increase appreciably from Kingman to Kiritimati (Table 1). Increase in nutrient delivery rates would favor macroalgal growth relative to coral growth [60], or enhance fish (predominately planktivore) population sizes [61].

Historical reports and biogeographical comparisons, however, suggest that differences in oceanography alone are insufficient to cause the observed differences in fish and benthic communities among the atolls. The earliest historical descriptions of Kiritimati and Tabuaeran document an enormous abundance of sharks and other large fishes (Table S2) that persisted until the early to mid 20<sup>th</sup> century when declines became apparent. As recently as 1997, fish biomass at Kiritimati was double that observed in our study and was comprised of over 30% top predators [62], suggesting that large declines in the fish assemblage have occurred within just ten years as the human population rapidly increased due to deliberate relocation (Supplemental Data S1; Figure S1). Thus, the low fish biomass at these atolls most likely is due to fishing here, as in many places elsewhere [39,63,64].

Coral cover also decreased greatly at Kiritimati, Tabuaeran, and Palmyra between 1997 and our surveys in 2005 [61,65], but remained relatively constant at Kingman [61]. These declines likely reflect effects of high bleaching stress associated with sustained periods of anomalously high seawater temperatures in the late 1990s and early 2000s (Table 1) [61]. However, comparison with atolls near to the northern Line Islands suggest that human activities have greatly exacerbated the effects of high temperature at Tabuaeran and Kiritimati. Uninhabited and protected Jarvis, Howland, and Baker atolls lie 1° to 1.6° to the south of Kiritimati and exhibit relatively higher chlorophyll *a* concentrations and more frequent SST anomalies associated with coral bleaching (*i.e.*, NOAA Level 2 events; Table 1) [61,66]. Extensive episodes of upwelling also have been documented in detail at Jarvis [67]. Nevertheless, these equatorial atolls support very high biomass of fishes dominated by apex predators and high live coral cover comparable to those at Kingman (Table 1) [61]. Because of the remoteness of the atolls, monitoring of the biota has been infrequent and the history of coral bleaching has not been studied in detail. But the fact that the coral cover at these atolls has remained high despite such thermal stress suggests that the corals have survived or have recovered from warm-water anomalies (consistent with models of ecological resistance or resilience, respectively) at uninhabited Jarvis, Howland and Baker, but not at Kiritimati and Tabuaeran where fishing effort and human population density are the highest in the region.

Combining data from our study with those from Jarvis, Howland and Baker (Table 1), we found no significant correlation between fish biomass or coral cover versus any of the oceanographic parameters (Frequency of Thermal Stress, SST, DHW, Chl *a*, DIN, SRP). Most strikingly, there were no correlations between fish or corals and either the maximum number of DHW per year (Pearson Correlation

for fish:  $r = 0.44$ ,  $t = 1.08$ ,  $p = 0.84$ ; and for coral:  $r = 0.28$ ,  $t = 0.65$ ,  $p = 0.73$ ) or the frequency of thermal stress (NOAA level 2) that is commonly associated with serious coral bleaching events (fish:  $r = 0.23$ ,  $t = 0.52$ ,  $p = 0.69$ ; coral:  $r = 0.49$ ,  $t = 1.26$ ,  $p = 0.87$ ).

It is interesting that the concentration of nutrients (DIN and SRP) around all four of the northern Line Islands surveyed are higher than the hypothesized thresholds for outbreaks of macroalgal blooms [68] and are well within the range for polluted Florida Bay (*e.g.*, 0.7 to 10.7  $\mu\text{Mol}$  for DIN) [69]. Nevertheless, macroalgal blooms are absent at Kingman and Palmyra, which suggests that grazing activity on unpopulated reefs may control macroalgal abundance even at high inorganic nutrient concentrations.

We propose a model of reef degradation consistent with our data and analogous to Birkeland's concept of ratcheting down coral reefs, where multiple, interacting effects of anthropogenic and natural stressors lead to the demise of reef health [70,71]. In the northern Line Islands the combined effects of local human impacts (predominantly fishing), global human impacts (global warming), and natural oceanographic variation have contributed to the observed patterns. The death of many corals from Kiritimati and Tabuaeran between 1997 and 2005 was most certainly caused by warm water events in the late 1990s and early 2000s. However, the magnitude of and the lack of recovery from these mortality events was likely influenced by anthropogenic changes in the local reef community. Based on the historical and geographic comparisons outlined above, we suggest that reef degradation in the northern Line Islands started because of and was enhanced by local anthropogenic stress.

## Conclusion

Fish biomass and the proportion of apex predators at Kingman atoll are greater than previously described from any coral reef ecosystem [15,27] and was associated with high cover of reef-building corals and crustose coralline algae, abundant coral recruits, and low levels of coral disease. More detailed, long-term observations and experiments are needed to more firmly establish cause and effect. But it is already apparent that reef communities with relatively intact food webs like those at Kingman, Jarvis, Howland, and Baker are the best available baselines for Pacific reefs, with biomass of top predators higher than in other well-protected areas such as the Great Barrier Reef in Australia [72], Kenya [15], or the Northwestern Hawaiian Islands [23]. Moreover, these uninhabited reefs appear to retain greater capacity to survive or recover from major episodes of coral disease or bleaching, whereas reefs with highly altered food webs like Tabuaeran and Kiritimati do not.

Thus, local protection from overfishing and pollution may enhance ecosystem resilience to warm episodes and coral bleaching that result from global warming. To test this we need to determine how do coral recruitment, growth, and survivorship respond to changes in local community structure due to fishing, and how do these responses interact with episodes of warming measured by DHW. We also need to determine how fish productivity, *i.e.*, the key currency of fisheries management, varies with changes in food web structure such as those observed between Kingman and Kiritimati. The only way to answer these questions is by investigation of reefs like the northern Line Islands that have remained remarkably intact in comparison to the global norm. They are among the only baselines that remain.

## Materials and Methods

Surveys of macroflora and macrofauna were conducted using SCUBA-assisted coral reef assessment techniques in August–September, 2005. Approximately 20 km of coastline were

surveyed per atoll in the semi-exposed leeward fore reef habitats between 10–12 m depths. We chose to compare the leeward side of the atolls for logistical reasons and because human activities are concentrated on the leeward side near human settlements and anchorages. However, windward and leeward are not as clearly defined around the smaller Palmyra and Kingman atolls so we sampled around the entirety of these atolls to maintain consistent areal coverage within the specified depth range.

### Reef Fishes

Surveys were conducted by two teams of paired divers, with four divers (ED, AF, ES, SS) rotating between teams to distribute individual biases. Stations were spaced about a kilometer apart at random locations. At each station, one team of tandem-paired divers tallied all fishes as they were encountered within fixed-length (25-m) strip transects whose widths differed depending on direction of swim. Transect bearings were determined haphazardly along isobaths (between 10 and 12 m depth). Each diver was responsible for one-half of the areas surveyed, as follows: large-bodied vagile fishes  $\geq 20$  cm total length (TL) were tallied within an 8-m wide strip (two 4-m wide swaths separated by 1 m) surveyed on an initial “swim-out” as the transect line was laid. Small-bodied, less vagile and more site-attached fish  $< 20$  cm TL were tallied within a 4-m wide strip surveyed on the return swim back along the laid transect line. Fishes were recorded by species or lowest recognizable taxon. Tallies were binned by 5-cm TL class. Three transects, each separated by about 10-m distance from its neighbor, were surveyed at each station. Thus, at each station, the densities of large-bodied fishes were estimated within a 600 m<sup>2</sup> (3×25×8 m) area, and the densities of small fishes within a 300 m<sup>2</sup> (3×25×4 m) area. Additional species richness data were recorded to complement those recorded on transects. Species presence was tallied within 3,000 m<sup>2</sup> (100-m long by 30-m wide) areas searched by 1-way zigzag swims centered on the transect lines.

Transects provided the input to estimates of species- and size-specific numerical densities. Various published [73], unpublished (JD Parrish, US Geological Survey, Hawaii Cooperative Fishery Research Unit), and web-based [33] sources provided the length-weight regression parameters necessary for converting numbers to biomass. Density and biomass were standardized to one square meter. No differences in total fish biomass were apparent among the various teams of paired divers (2-way ANOVA—team effect:  $F_{3, 93} = 1.66$ ,  $p = 0.18$ ; team×atoll interaction effect:  $F_{9, 82} = 2.09$ ,  $p = 0.04$  ns). Second, there were no differences among teams in recorded composition of fishes; species density (richness per station) averaged  $100 \pm 5$  species per station for all teams (2-way ANOVA—team effect:  $F_{4, 93} = 2.24$ ,  $p = 0.07$ ; team×atoll effect:  $F_{10, 83} = 1.65$ ,  $p = 0.11$ ).

Numerical density and biomass of major fish trophic groups were compared among reefs by k-sample median tests [74] and ANOVA. Analysis routines were applied using SAS v. 9.

### Corals

Corals were surveyed in four ways to characterize their abundance, diversity, size distribution, and health. (i) Coral density and size was measured in two 25×2 m transects (100 m<sup>2</sup> total area) per site; the identity (at least to genus level) and size of all corals with colony center within 1m of the transect were measured. (ii) Percent cover was estimated using photoquadrats (see *Benthic cover* below). (iii) We quantified coral health by measuring pathologies and magnitude of disease, bleaching, and/or drastic predatory damage recorded (see *Coral disease* below). (iv) In the 60×20 m area including the transects, divers (JM and DO) swam to develop a site-specific diversity list to the species level.

### Benthic Cover

Quantitative assessments of the benthos were made using the photoquadrat method [75]. At each site two 25 m transects were placed on the benthos parallel to shore, 25 m apart and at a constant depth of 10–12 m. Ten points were randomly selected and surveyed per transect. At each point a photograph was taken using an Olympus 7070 digital camera that was connected to a quadpod (1 m high) and a frame (0.9×0.6 m or 0.54 m<sup>2</sup>). During surveys notes were made for each quadrat and collections were made for organisms that were unidentifiable in the field. Upon return to shore all photographs were edited using Adobe Photoshop v 7.0. Image analysis was completed using the program Photogrid 1.0. For each photograph 100 points were randomly generated and the organism under each point was identified. A total of 10 benthic sites were surveyed for each atoll. All organisms were identified to the finest level of resolution possible (genus level for hard and soft corals, functional group for algal turfs and crustose coralline algae, and species level for macroalgae and macroinvertebrates when possible).

The structure of the benthic community was compared among reefs by PERMANOVA [76], and the cover of individual functional groups of organisms by ANOVA. To test for differences in the density of coral recruits (small colonies [1–5 cm] of *Acropora*, *Pocillopora* and fungiids, taxa that could be definitively identified as recruits) we performed ANOVA on log-transformed data. To compare the standardized coral recruit density among atolls (densities of recruits standardized by the mean atoll-specific total densities of colonies  $< 5$  cm) we performed ANOVA on arcsine square root transformed data.

### Coral Disease

Surveys describing the health status of corals were conducted on two, 2×20 m belt transects at 10 sites on each of the four atolls. Coral colonies were examined for gross morphological signs of stressors and placed into either a known disease, compromised or predation categories. The disease categories included, White syndrome, Skeletal Eroding Band, Brown Band, Black Band and other cyanobacteria (descriptions found in [77]). Compromised categories included signs of coral tissue necrosis that are not a formally recognized disease state (such as a degenerative syndrome, where partial mortality was caused by a combination of sedimentation, increased mucus production, and the presence of low numbers of cyanobacteria); algal interactions with corals that cause tissue bleaching and erosion; bleached white patches, which were bleached but had intact tissue on coral colonies and pink coloration (which suggests a stress response by the coral, mostly present in the family Poritidae). Predator feeding scars from *Drupella* species and *Acanthaster planci* were recorded, but were not analyzed in this study. Coral showing signs of either disease or compromised health were used to analyze the relationship between the number of unhealthy and healthy corals on coral reefs associated with different levels of human activity. Because of logistical constraints, surveys describing the health status of the corals and those enumerating total coral numbers were conducted by separate researchers on the same transects. The transects focusing on disease signs were 5 m shorter than transects conducted to enumerate the total number of corals at each site (i.e. the first 20m of the 25m benthic transect). Therefore, the prevalence of unhealthy corals was calculated on a m<sup>2</sup> basis by dividing the number of unhealthy colonies by the total number of coral colonies. To test for differences in the incidence of coral disease among atolls we used a non-parametric Kruskal-Wallis test.

### Mobile and sessile (non-coral) macroinvertebrates

Population density and species richness of mobile macroinvertebrates were determined within one 60×2 m belt transect at each of the 10 benthic stations on each atoll (except at the first station in Kiritimati, where a 1m wide transect was used). Mobile macroinvertebrates were defined as species that live unattached and grow to >5 cm, and comprised 24 species of echinoderms (asteroids, echinoids, holothurioids), mollusks (gastropods, bivalves, and cephalopods), and crustaceans (lobsters). Only visible (exposed or partially exposed) animals were counted. Species richness of sessile macroinvertebrates other than scleractinian corals was determined around the same belt transect used for mobile macroinvertebrates. Both species within the belt transect, as well as those encountered around the belt transect within a 30-minute total search time were recorded. Sessile macroinvertebrates were defined as species that live attached and grow to >5 cm, and comprised 21 species and forms of anthozoans (octocorals, zoanthids, actiniarians, and antipatharians), and sponges. *Simularia* soft corals were differentiated into morphospecies based on growth form; all other anthozoans and sponges were differentiated to species. Only visible (exposed or partially exposed) animals were recorded. Numerical density of major species and taxonomic groups were compared among reefs by k-sample median tests [74].

### Structure of coral reef communities

To explore the gradients in coral reef community structure (abundance of functional groups) among sites and atolls we performed a principal components analysis. Data were normalized, following arcsine square root transformation for benthic percent cover and natural logarithm transformation for fish biomass, and standardized.

### Species richness of fishes and corals

Total species richness estimates were compared among atolls. For fishes and for corals, richness was estimated using presence/absence data from each station. Incidence-based coverage estimators were used to account for differing abundances across atolls, providing an extrapolated estimate of the total number of species present in the sampled area [78].

### Oceanographic data

Mean and standard deviation of sea surface temperature (SST) were calculated from monthly mean SST estimates for the 50km water pixel including each atoll using US National Oceanic and Atmospheric Administration (NOAA) Coral Reef Watch data from 1985–2005.

The annual accumulation of degree heating weeks (DHW) is determined from these twice-weekly SST data. One DHW is equal to one week of SSTs that are one degree greater than the mean temperature of the warmest month in the climatology (i.e., the historical mean for July in most of the Line and Phoenix Islands). The total DHW accumulation for a given year is the accumulation of positive SST anomalies over a rolling 12 week time period; only anomalies in excess of 1°C were included, because smaller SST spikes are believed to be insufficient to cause stress on corals (i.e., consecutive weekly anomalies of 1.0, 1.5 and 0.8 results in a total DHW value of 2.5, because the third value is less than one). Notably, El Niño Southern Oscillation (ENSO) anomalies are the common cause of maximum DHW episodes. Because ENSO events typically occur during the boreal winter (December–January) in the region, we calculated the annual maximum number of DHW for 12 mo periods from July in one year until June in the following year.

Bleaching Alert Level 2 is the highest level of coral bleaching warning issued by NOAA and is defined as a period with  $\geq 8$  maximum DHW. We computed the frequency of Level 2 bleaching events as the percentage of years from 1985–2005 with maximum DHW at or above this threshold. We present individually the years since 1990 that have reached this threshold.

The chlorophyll *a* data used in this study were acquired using the GES-DISC Interactive Online Visualization ANd aNalysis Infrastructure (Giovanni) as part of the NASA's Goddard Earth Sciences (GES) Data and Information Services Center (DISC). The OBP (Ocean Biology Processing Group) MODIS/Aqua monthly global 9-km products were subdivided into 2°×2° boxes surrounding each atoll creating area-averaged values for each box. Monthly chlorophyll *a* and standard deviation products were averaged over the available time period (July 2002 through October 2006) to calculate mean chlorophyll *a* concentration (mg/m<sup>3</sup>) and standard deviations for each of the atolls. Kingman and Palmyra were calculated using the same 2°×2° bin because of their proximity to each other; Howland and Baker were calculated the same way.

### Water chemistry

At each atoll water samples were collected from 4 (5 at Palmyra) evenly distributed stations at approximately the same time in the morning on each occasion. At each station a total of 8 samples were taken using diver-adapted polycarbonate 2-liter Niskin bottles. Water was transferred back to the OR/V White Holly and processed within 1 hour of collection. The Niskin bottles were connected directly to a positive-pressure filtration system minimizing external contamination. All fixtures were made from polycarbonate or silicone. Acid washed tubing and filter cassettes were flushed with 200 ml of the sampled water before sample collection began. Samples were collected under positive pressure by applying 3 PSI of pressure to the Niskin bottles using a SCUBA tank.

Water was collected for inorganic nutrient analysis by filtering through 0.2  $\mu$ m Nuclepore Track-Etch membrane filters (Whatman). Fifteen ml of filtrate was collected in HDPE scintillation vials with cone-shaped plastic lined lids (Fisher Scientific) after rinsing both the bottles and lids 3 times with filtrate, and then stored at –20°C. Analysis of inorganic nutrients (nitrate, nitrite; ammonium and ortho-phosphate) concentrations was carried out by the Marine Science Institute's Analytical Lab, University of California, Santa Barbara, California, USA using a QuikChem 8000 flow injection analyzer (Lachat Instruments, Wisconsin, USA). Data for nitrogen are presented as dissolved inorganic nitrogen (DIN), i.e., the total of ammonium and Nox (i.e., nitrate+nitrite). Data for phosphorus are presented as total soluble reactive phosphorus (SRP). Means and standard errors of nutrient concentrations were computed across stations for each atoll.

Water was also collected for chlorophyll *a* analysis. Triplicate 500 mL samples were filtered onto 25 mm GF/F (Whatman) and immediately stored at –80°C. Within two months, filters were extracted in absolute methanol for 1 h [79] and chlorophyll *a* concentrations measured on a Turner Designs (model 10–005R) fluorometer. Means for each station and standard errors among stations were determined for each atoll.

### Supporting Information

**Supplemental Data S1** The gradient of human disturbance in the northern Line Islands: Population, fishing, and waste. Found at: doi:10.1371/journal.pone.0001548.s001 (0.05 MB DOC)

**Table S1** Aquarium reef fish catch for export at Kiritimati (April–December, 2005).

Found at: doi:10.1371/journal.pone.0001548.s002 (0.03 MB DOC)

**Table S2** Historical accounts of shark abundance in the Line Islands.

Found at: doi:10.1371/journal.pone.0001548.s003 (0.05 MB DOC)

**Table S3** Non-coral invertebrate abundance on the northern Line Islands.

Found at: doi:10.1371/journal.pone.0001548.s004 (0.12 MB DOC)

**Table S4** Non-coral invertebrate data analysis: Median test for comparison of species abundance between atolls.

Found at: doi:10.1371/journal.pone.0001548.s005 (0.05 MB DOC)

**Figure S1** Population data for the northern Line Islands

Found at: doi:10.1371/journal.pone.0001548.s006 (0.05 MB TIF)

**Figure S2** Total annual reef fish catch at Kiritimati

Found at: doi:10.1371/journal.pone.0001548.s007 (0.68 MB TIF)

**Figure S3** Annual Fish Catch for Export on Kiritimati

Found at: doi:10.1371/journal.pone.0001548.s008 (0.66 MB TIF)

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**Figure S4** Map of the central Pacific, including atolls of the Line and Phoenix Islands. Colors reflect mean sea surface temperatures for August 2005. Note the latitudinal gradient of temperature determined by meeting of the equatorial current and countercurrent

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## Author Contributions

Conceived and designed the experiments: JJ NK GP ES JS SS FR OP ED AF JM DO. Performed the experiments: GP ES JS SS FR ED OP ED AF MM JM DO. Analyzed the data: ES JS SS FR ED OP ED AF JM DO. Contributed reagents/materials/analysis tools: FR SD TK MR SW RS. Wrote the paper: JJ NK ES JS SS.

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# Microbial Ecology of Four Coral Atolls in the Northern Line Islands

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## Abstract

Microbes are key players in both healthy and degraded coral reefs. A combination of metagenomics, microscopy, culturing, and water chemistry were used to characterize microbial communities on four coral atolls in the Northern Line Islands, central Pacific. Kingman, a small uninhabited atoll which lies most northerly in the chain, had microbial and water chemistry characteristic of an open ocean ecosystem. On this atoll the microbial community was equally divided between autotrophs (mostly *Prochlorococcus* spp.) and heterotrophs. In contrast, Kiritimati, a large and populated (~5500 people) atoll, which is most southerly in the chain, had microbial and water chemistry characteristic of a near-shore environment. On Kiritimati, there were 10 times more microbial cells and virus-like particles in the water column and these microbes were dominated by heterotrophs, including a large percentage of potential pathogens. Culturable *Vibrios* were common only on Kiritimati. The benthic community on Kiritimati had the highest prevalence of coral disease and lowest coral cover. The middle atolls, Palmyra and Tabuaeran, had intermediate densities of microbes and viruses and higher percentages of autotrophic microbes than either Kingman or Kiritimati. The differences in microbial communities across atolls could reflect variation in 1) oceanographic and/or hydrographic conditions or 2) human impacts associated with land-use and fishing. The fact that historically Kingman and Kiritimati did not differ strongly in their fish or benthic communities (both had large numbers of sharks and high coral cover) suggest an anthropogenic component in the differences in the microbial communities. Kingman is one of the world's most pristine coral reefs, and this dataset should serve as a baseline for future studies of coral reef microbes. Obtaining the microbial data set, from atolls is particularly important given the association of microbes in the ongoing degradation of coral reef ecosystems worldwide.

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## Introduction

The roles of microbes, both Bacteria and Archaea, and viruses on coral reefs are just starting to be elucidated. Most studies concern microbes in the water column, although actual densities are much higher in the benthos [1]. Microbes may play an important role in the nutrition of reef organisms. For example, the number of microbes in the water column declines from the windward to leeward (forereef to backreef) areas of coral reefs [2], suggesting ingestion by coral reef organisms [3–5]. Similarly, decreasing densities of bacteria have also been documented within the vertical structure of a coral reef, with the over-lying water column containing approximately 4.5 times the amount of bacteria compared with the water within crevices of the coral reef structure [6].

Our ability to understand these microbes has increased greatly with the development of molecular and genomic approaches that provide a far more accurate picture of community composition and activities. In the marine environment molecular techniques have identified new organisms and new metabolic processes [7]. For coral reefs, molecular techniques, such as 16S rDNA analysis has identified that microbial communities associated with corals are diverse and develop both species specific [8], and generalist associations [9]. These molecular techniques have also revealed the etiological agents of diseases of coral reef organisms, such as, corals [10] and sponges [11,12]. In some cases the etiological agents are not specific to corals, but infect multiple and distinctive marine organisms [13], leading to difficulties in identify causative agents of the increasing number coral diseases that are described

[14]. The lack of identified pathogens suggests opportunistic bacterial infections or hard-to-culture pathogens (e.g., viruses) are important mediators of coral disease. Because of these difficulties, metagenomics, which allows the entire genome of all the micro-organisms within an environment to be sampled rapidly [15], may be required to describe microbial associations on coral reefs and how they change with environmental fluctuations and anthropogenic activities. Metagenomic studies are not restricted by targeting single gene regions, but provide information on all genomic regions, enabling both taxonomic descriptions and potential metabolic functions of the micro-organisms within an environment to be described [16]. For coral reefs, a comprehensive evaluation of the microbial and viral community may be particularly important because coral diseases are an increasing factor in the global collapse of reef ecosystems [14,17].

In this study, the coral reef microbial communities associated with four coral atolls in the Northern Line Islands (central Pacific) were surveyed. These atolls are of interest because although they are relatively close to each other (750 km), they span an array of oceanographic conditions and are variably impacted by human activities. One of the atolls is pristine with respect to local anthropogenic effects, and thus provides an important microbial baseline against which other reefs can be compared. Microbial and viral abundances were complemented with metagenomic analyses of these communities. Coral cover and disease prevalence were also measured to determine if there were correlations between microbial communities and coral health. A companion study complements the microbial data by measuring functional changes of the fish and benthic communities [18].

## Materials and Methods

### Study sites

Surveys were conducted on four atolls in the Northern Line Islands in the central Pacific (Figure 1; [18]). The atolls are separated by ~750 km and span a gradient in oceanic productivity and climate. The level of rainfall declines from north to south [19], whereas oceanic productivity declines from south to north.

Two atolls, Kingman and Palmyra, are part of the US national refuge system and have little or no local anthropogenic impacts. The most northern atoll, Kingman, is uninhabited and has only a few emergent sand bars (<0.1 km<sup>2</sup>), and the reef crest surrounds a relatively large lagoon (60 km<sup>2</sup>). As such, it is expected to have a high flushing rate and no terrestrial influence of any kind (e.g., human sewage, bird guano, agricultural runoff). Palmyra has ~7 km<sup>2</sup> of land and a relatively small lagoon (15 km<sup>2</sup>) that was extensively remodeled during World War II. There are only ~20 people on Palmyra at any one time, and sewage is treated and contained. Seabirds are numerous and a potential source of nitrogen, but guano was never mined on Palmyra because it does not accumulate in significant quantities. Ammunition dumps are also potential sources of nitrogen compounds. There are a number of small wrecks and former military structures, which may increase local levels of iron. Shark finning occurred on Palmyra in the past.

The remaining two atolls, Tabuaeran and Kiritimati, are inhabited and part of the Republic of Kiribati. Tabuaeran has 34 km<sup>2</sup> of land and a 110 km<sup>2</sup> lagoon; a natural passage on the west side of the island was widened by blasting of coral heads by the British, in the 1890s. There are ~2500 people and a large wreck just north of the lagoon passage. All that visually remains of this wreck are a number of large boilers. Kiritimati has 390 km<sup>2</sup> of land and 324 km<sup>2</sup> of lagoon, and ~5500 people. Human sewage is untreated on both Tabuaeran and Kiritimati and there was no evidence of any traditional management of sewage. There are

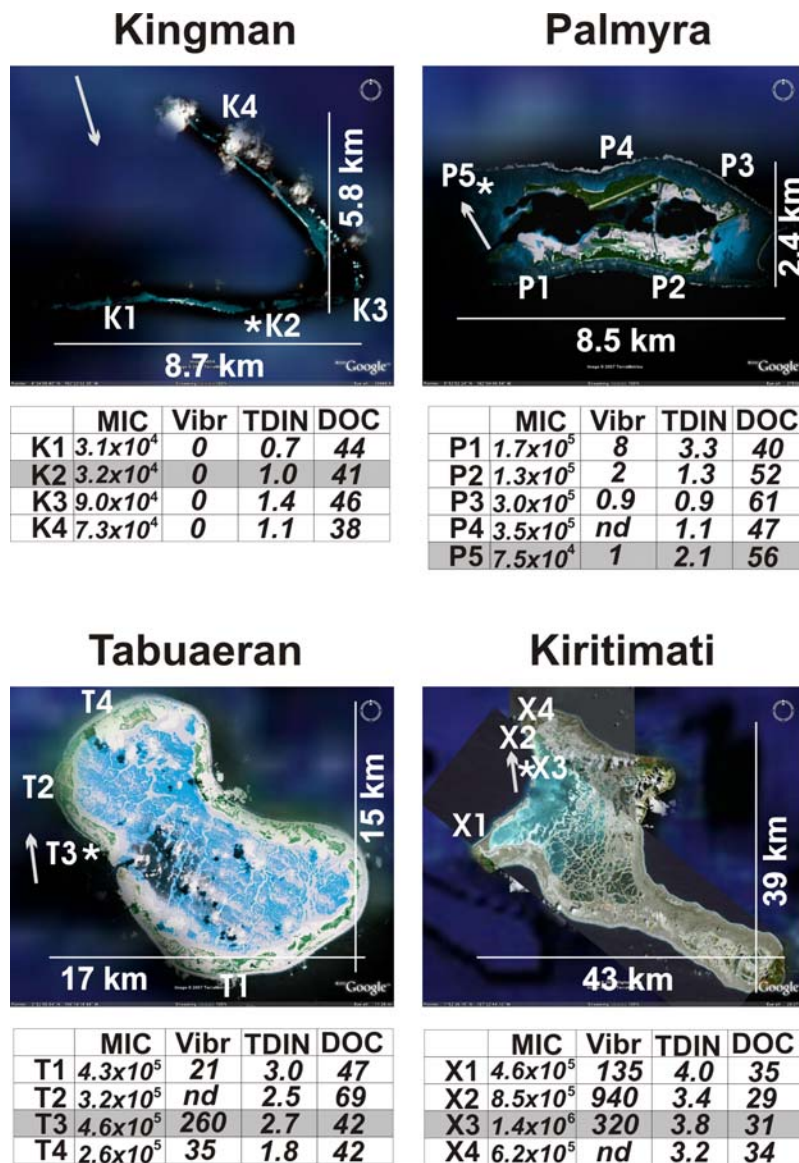
some septic tanks associated with the hotel and larger buildings on Kiritimati; many of these leak, and combined with the untreated sewage, there are a number sanitation problems associated with the island's water supply. Recently installed composting toilets are often not used because of local customs and beliefs ([http://www.unep.or.jp/ietc/publications/techpublications/techpub-15/3-8IlandPacific/8-11-2\\_1.asp](http://www.unep.or.jp/ietc/publications/techpublications/techpub-15/3-8IlandPacific/8-11-2_1.asp)). There have been several agriculture initiatives on Tabuaeran and Kiritimati, including seaweed and coconuts. Guano was mined on Kiritimati in the 1850's and 1860's, but it is not known how much mining actually took place; mining stopped in 1866 because it was unproductive. Atomic bomb testing was conducted on Kiritimati between 1957 and 1962 by both the British and American military. Kiritimati has a series of lagoons, a large main one and then many smaller lagoons that are both connected and unconnected to the main lagoon. The highest fluctuations in physio-chemical properties occurs in the unconnected lagoons [20,21], however the main lagoon has salinities and pH similar to seawater. Dissolved oxygen levels were low in the main lagoon and found to increase only in close proximity to settlements, suggesting eutrophication of small areas [20]. Microbial activity in the lagoon sediments was high, but similar to levels in other Pacific lagoons [21].

### Survey overview

Microbial communities were surveyed in the coral reef waters on each of the four atolls at 10–12 m depth (<400 m from shore), between August 4<sup>th</sup> and September 6<sup>th</sup> 2005. We used the following approaches: 1) Quantification of Bacteria and Archaea (microbes), virus-like particles (VLPs), and protists using direct counts on water collected from above the reef substratum, 2) Abundances of culturable *Vibrio* spp. determined by counting colony forming units (cfu) on thiosulfate citrate bile sucrose plates (TCBS), and 3) Taxonomical and metabolic potential of the microbial and viral communities using metagenomic analyses. In addition, we characterized the coral community (percent cover, disease prevalence) and the water chemistry [concentrations of total dissolved inorganic nitrogen compounds (TDIN: ammonium, nitrate, nitrite), phosphate, and dissolved organic carbon (DOC)].

At each atoll we used the same sampling strategy (one site for metagenomic analyses, four to five sites for other microbial and water chemistry samples, 10–12 sites to characterize the benthic community, ~30 sites (separated by 2 km) to characterize the fish community). The general sampling scheme was centered at the leeward side and worked out in both directions around the island (Figure 1, see Sandin et al [18]; further details below). Because of the differing rates at which the fish, benthic, and microbial surveys could be conducted, not all groups were characterized at each site. Thus about 50% of total sites sampled for coral cover and other benthic properties were also microbiologically characterized.

For the metagenome samples, areas underneath the lagoon currents were targeted because: 1) It was expected that these areas would be the most likely to show signs of human disturbance, 2) Time and resources limited the survey to one microbial and one viral metagenome per atoll, and 3) This limited sampling meant that it was necessary to target an area of the reef that had similar hydrological characters, and lagoon currents are a relatively constant feature of coral reefs. The lagoons of Palmyra, Tabuaeran, and Kiritimati tend to flush in a northerly direction. The samples for the metagenomes were taken from benthic sites that are flushed with the lagoonal waters. In the case of Kingman, the water flows over the reef. The prevailing current, during the cruise was from the north to south (as determined with a float), so this metagenome sample was taken on one of the gaps on the southside of the atoll (Figure 1).



**Figure 1. Maps of the sites surveyed on the four Northern Line Island atolls.** The locations for the water chemistry, microbe/viral direct counts, and *Vibrio* spp. culturing are indicated with the first letter of the atoll name (X for Kiritimati sites) and sequentially numbered. The sites for the metagenomes are labeled with an \*. Coral cover, fish counts, and other macro-organism data were sampled at all of these sites, as well as additional sites [18]. The prevailing current is shown as a grey arrow. **MIC** = number of microbes per ml; **Vibr** = number of culturable *Vibrio* spp. on TCBS plates per ml; **DOC** = dissolved organic carbon in  $\mu\text{M}$ ; **TDIN** = total dissolved inorganic nitrogen in  $\mu\text{M}$  (nitrite and nitrate, and ammonium). Maps were taken from Google Earth.

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### Direct counts of Bacteria, Archaea and virus-like particles

The numbers of microbes (both Bacteria and Archaea) and virus-like particles (VLPs) in the water column were determined via direct counts using epifluorescent microscopy. Pre-washed diver-adapted polycarbonate Niskin bottles were used to sample the water at each site. Each Niskin bottle sampled 2 liters of seawater, which was used for the direct counts, culturing, and water chemistry. Four of the bottles were collected from the reef surface ( $\sim 10$  m depth), two bottles were collected 25 cm above the benthos, and two bottles were collected 500 cm above the benthos. No statistical difference was found between counts taken at various depths ( $F_{32} = 0.321$ ,  $P = 1$  for microbes and  $F_{32} = 0.320$ ,  $P = 1.0$  for VLPs), therefore samples were analyzed at the site level. The counts were conducted on 2 or 8 ml of sea water (two

concentrations were prepared to ensure that we obtained countable slides). The samples were fixed with electron microscopy-grade paraformaldehyde (4% final concentration) and stained with SYBR Gold ( $1 \times$  final concentration; formally Molecular Probes, Inc., now Invitrogen, Solana Beach, CA) and filtered onto  $0.02 \mu\text{m}$  Anodisc filters (Whatman, Inc, Florham Park, NJ), mounted on glass slides and directly counted by epifluorescence microscopy. Cells and VLPs were counted ( $>200$  per sample) in 10 fields selected at random. The microbes and VLPs counts were log transformed and compared using an unbalanced multivariate analysis of variance with sites nested within atolls. Normality and heterogeneity were tested using Kolmogorov-Smirnov and Levene tests, respectively. Atoll pairings were tested using a Wilcoxon one-sided analysis.

## Enumeration of protests

The protist samples were counted from 100 ml of seawater taken from each of the Niskin bottles, fixed with Lugol's solution and formalin, de-stained with sodium thiosulfate, filtered onto 0.6  $\mu\text{m}$  polycarbonate membranes (Millipore, Billerica, MA) and stored at  $-20^{\circ}\text{C}$ . Filters were stained for three minutes with 400  $\mu\text{l}$  DAPI (1  $\mu\text{g ml}^{-1}$ ; Sigma), rinsed with deionized water and mounted onto glass slides using Vectashield (Vector Laboratories, Inc.; Burlingame, CA) [22]. Filters were observed via epifluorescence microscopy with a BX51 microscope (Olympus America, Inc) and protist abundances were determined based on average counts of  $\sim 20$  fields per filter. Pigmented and non-pigmented protists were differentiated using the TRITC band excitation filter set (excitation  $\sim 550$  nm; emission  $\sim 600$  nm). Pigmented protists were classified as autotrophic-mixotrophic and non-pigmented protists as strictly heterotrophic. Protist count data were normally distributed and homogenous and therefore were not transformed (tested as described above). An unbalanced ANOVA with sites nested within atolls was used to analyze the data, and Tukey's post-hoc test was used to identify the difference between atolls.

## Microbial and viral metagenomes

A sample of approximately 150 l of seawater was collected at one site per atoll (Figure 1). The water for the metagenomes was collected from below the boundary layer (in crevices and against the benthos) to avoid confounding problems with the water column. The sampling was conducted at the same time of day to help minimize diurnal effects. The water was collected from over  $\sim 20$   $\text{m}^2$  of reef using a modified bilge pump connected to low density polyethylene (LDPE) collapsible bags (19 l; Cole-Parmer, Vernon Hills, IL; Figure S1). The containers were transported to the surface and the research vessel within two hours of collection, thereby reducing potential *in situ* community changes. To remove potential sources of DNA contamination, containers, bilge pumps, and tubing were washed once with 10% bleach, three times with freshwater, and once with 100 kDa filtered seawater prior to sampling.

Two size fractions were prepared for the metagenomic analysis from the seawater samples: 1) A large fraction containing mostly microbes, some small eukaryotes (such as dinoflagellates and protists), and a few VLPs, and 2) a small fraction containing mostly VLPs and some small microbes. To obtain these fractions the seawater was processed through a series of filters. The large eukaryotes were removed by filtering the entire sample through 100  $\mu\text{m}$  Nitex, into a barrel lined with a clean, high-density polyethylene bag. The filtrate was then concentrated to  $\sim 500$  ml on a 100 kDa tangential flow filter (TFF), which captured the unicellular eukaryotes, microbes and VLPs (i.e., the water was removed). During the filtration, pressures were kept below 0.6 bar (10 psi) to ensure that the viruses were not destroyed. The concentrated sample was then passed through 0.45  $\mu\text{m}$  Sterivex filters (Millipore, Inc) using a 50 ml syringe. In this step, the large metagenomic fraction consisting of microbial cells was caught on the filter (microbiomes) and the filtrate was the small metagenomic fraction (viromes). All filtrations were performed on the research vessel, and the samples were stored for further processing in the laboratory at SDSU. The Sterivex filters were frozen at  $-80^{\circ}\text{C}$ . The 0.45  $\mu\text{m}$  filtrates (i.e., the virome) were extracted with chloroform to kill any residual cells (10% vol:vol; most viruses are resistant to chloroform) and stored at  $4^{\circ}\text{C}$ .

The DNA for the microbiomes was isolated from the Sterivex filters by removing the filter membranes and performing DNA extractions using a bead-beating protocol (MoBio, Carlsbad CA). The DNA obtained was amplified with Genomiphi (GE Healthcare Life Sciences, Inc, Piscataway, NJ) in six to eight 18-

hour reactions [23–28]. The reactions were pooled and purified using silica columns (Qiagen Inc, Valencia, CA). The DNA was then precipitated with ethanol and re-suspended in water at a concentration of approximately 300  $\text{ng } \mu\text{l}^{-1}$ .

The viruses in the small metagenomic fractions (i.e., 0.45  $\mu\text{m}$  filtrate treated with chloroform) were purified using cesium chloride (CsCl) step gradients to remove free DNA and any cellular material [29,30]. Viral DNA was isolated using CTAB/phenol:chloroform extractions and amplified in six to eight 18-hour Genomiphi reactions. These reactions were pooled and purified using silica columns (Qiagen Inc, Valencia, CA). The DNA was then precipitated with ethanol and re-suspended in water at a concentration of approximately 300  $\text{ng } \mu\text{l}^{-1}$ .

Both the virome and microbiome DNAs were sequenced at 454 Life Sciences (Branford, CT) using their parallel pyrosequencing approach.

## Initial bioinformatics on metagenomes

The DNA sequences generated by 454 Life Sciences, Inc, were analyzed *without* assembly. This approach simplifies the statistical analysis and avoids problems with chimera assemblies. Thus, these sequences represent environmental gene tags [31]—short fragments of genes that are found in the different samples. To characterize these sequences, several independent approaches were taken. In the first approach, the sequences were compared to the extant sequence libraries using the Basic Local Alignment Search Tool (BLAST) algorithm. Several boutique databases were used for rapid comparisons and categorization of the DNA sequences. All sequences were compared to the 16S ribosomal DNA database (version 9) available from <http://rdp.cme.msu.edu/>; the Phage Genome Database and the viral genome database <http://phage.sdsu.edu/phage/>; the European Ribosomal DNA database (<http://www.psb.ugent.be/rRNA/>); and the ACLAME (A Classification of Genetic Mobile Elements) database (<http://aclame.ulb.ac.be/>) [32–35]. Results from these analyses are all available at the Line Islands section of our website <http://scums.sdsu.edu>. Sequences were also compared to the SEED platform [36,37] using the BLASTX algorithm [38]. This database contains the protein sequences from all the available complete and draft genomes. The comparisons were run on the Life Sciences Gateway to the Teragrid (Judson, Edwards, Papka, and Stevens, in preparation). The number of sequences obtained from the 454 pyrosequencing and the number that showed significant similarities are provided in Supplementary Table S1.

The metagenomic sequence fragments with significant similarities (E-value  $\geq 0.01$ ) to the SEED platform (<http://metagenomics.theseed.org/>) were assigned functions based on their closest similarity. This approach allows rapid assessment of the metabolic potential of the sample and provide reliable taxonomic and functional assessments [29,30,39].

## Taxonomy and guild assignments

Taxonomical assignments of uncultured microbes are routinely based on sequence data. In the case of microbial metagenomes, this is done in two ways: 1) closest hits to 16S rDNAs, and 2) closest hits to any sequence from a known organism. In the former case, it is relatively easy to just compare the metagenome results to those obtained from traditional 16S rDNA surveys. Using this approach, it has been shown that both qualitatively and quantitatively, the 16S rDNA genes in metagenomes are very similar to those found by 16S rDNA sequencing [39]. This is true regardless of whether the metagenome is made by cloning and Sanger sequencing or by the cloneless 454 pyrosequencing approach. In the latter case, the shorter sequences ( $\sim 100$  bp)

yield the same overall result as the longer Sanger sequences. Though less established than the 16S rDNA approach, taxonomical assignment of metagenomic sequence fragments based on their closest hits to any sequence from a known organism is accurate and informative, and much more metagenomic data are utilized in the analysis. Ground-truthing in this case has been done by a variety of comparisons to coding genes, as well as to cloned 16S rDNA libraries [39]. This approach has also been validated for 454 pyrosequencing data [40].

Both 16S rDNA cloning approaches and metagenomics suffer from the fact that the microbes are not in culture. Very closely related microbes can have quite different phenotypes based on just a few genes (e.g., exotoxins). Therefore, assignment of microbes to guilds based on uncultured data needs to be qualified. For this study we chose to assign the metagenomic fragments to autotrophs, heterotrophs, and potential pathogens (see below for details). While there might be some overlap between these guilds, the results are very similar whether just the 16S rDNA assignments are considered or functional assignments are used. Although as noted above, small genetic differences can result in major guild differences, the methods were applied consistently across all the atolls so that bias associated with the method would be systematic in nature.

The “habitat” of each microbe with a genome in the SEED platform was manually curated from Bergey’s Manual of Determinative Bacteriology [41]. For example, cyanobacteria were assigned to the “autotroph” guild and SAR11 was assigned to the “heterotroph” guild. The “potential pathogen” guild consisted of metagenomic sequence fragments most closely related to the human pathogenic genera *Staphylococcus*, *Vibrio*, and *Escherichia*, the fish pathogens belonging to *Aeromonas*, and plant pathogens from the *Xylella* genera. A complete list of the organisms that we described as autotrophic, heterotrophic or potential pathogens is provided on the Line Islands section of our accompanying website at <http://scums.sdsu.edu> under the section titled “Bergey’s listing”. This categorization was used to estimate the proportion of microbes that could be described as belonging to one of these three groups on each atoll.

### SEED-based assignments of metabolic potential

The metabolic potential of each sample was identified by examining the similarities between the metagenomic sequence fragments and genes in metabolic subsystems. A metabolic subsystem is a group of genes that together form a metabolic function or pathway. The complete list of genes that was similar to metabolic subsystems for microbiomes is provided on <http://scums.sdsu.edu>. Descriptions of each of the subsystems are available from <http://www.theseed.org/>. The frequency that each metabolic subsystem was found at each atoll was visualized using a novel interactive web interface which color-codes the frequency to which each subsystem is found in each metagenome sample (<http://metagenomics.theseed.org/>).

Two different methods were used to calculate the statistical significance of the presence and absence of different metabolic subsystems in each sample: 1) The XIPE-TOTEC subsampling method [37], and 2) the G-test (a maximum likelihood test), neither of which depend on a normal distribution of the data. The two approaches identified which metabolic subsystems were statistically over-represented between the atolls (results are provided at <http://scums.sdsu.edu>). The analyses of the subsystems and accompanying statistics were used to calculate the seven most abundant subsystems that were different between Kingman and Kiritimati. Relative representation of each subsystem was calculated as the number of similarities to a particular subsystem divided by the total number of

similarities to any subsystem; using percentages allows comparisons between samples regardless of the number of sequences obtained from each pyrosequencing reaction.

### Bioinformatics of the viromes

The small fraction metagenomic libraries were compared to the known phage genomes in the phage database (v. 5; <http://phage.sdsu.edu/phage>). This database contains 510 phage genomes and was used to construct the latest version of the Phage Proteomic Tree [34,42]. To compare the relative numbers of phage hosts at each atoll, the incidence of each phage host genome per atoll was counted. Counts were normalized for the number of sequences per metagenome, enabling direct comparisons between metagenomes. The microbe that the phage infects was then compared against the “Bergey’s List” to describe the phage as either infecting an autotrophic, heterotrophic or potentially pathogenic bacteria identified as described above.

Two individual phage and prophage strains (*Escherichia coli*  $\Phi$  CP4-6 prophage and *Prochlorococcus marinus* SSMP4 phage) were used to provide information on the spread of sequence similarities across individual genomes. The coverage maps were constructed by creating a database with the phage or prophage genomes of interest and comparing the metagenomes against them by using TBLASTX. Sequences with E-values  $\leq 0.0001$  were binned into 1000 base pair windows along the reference genomes. The total number of sequence similarities within a window was calculated and divided by the total number of sequences for each metagenome, allowing comparisons between metagenomes.

### Pfam and GO term analysis

The metagenomic libraries were also analyzed using the Pfam database as a comparison to the SEED platform conclusions. To identify conserved protein domains in the metagenomes, all sequence fragments from a sample were translated into each of the six reading frames. To save computational time, translated sequences with an in-frame stop codon were excluded from further analysis. The remaining sequences were locally aligned to each Pfam family using the pHMM from the Pfam\_fs database (version 20.0). To search the Pfam\_fs database, hmmpfam was run on a computer cluster at the Center for Biotechnology (CeBiTec; Bielefeld University, Germany) [43]. The E-value cut-off was set to 0.01.

Conserved protein domains were categorized into functional groups according to the Gene Ontology (GO) [44]. For each identified domain, GO terms were obtained from its Pfam family description. A pair-wise comparison of all samples versus all samples was performed to identify overrepresented GO terms in a sample. To determine if a GO term was significantly overrepresented, the  $G_{adj}$ -test was employed under the null hypothesis  $H_0$ : there is no difference in the abundance of identified domains in the two samples to which the GO term was assigned. Values of G were adjusted by the Williams’ correction factor. The significance level was set to  $P < 0.05$ .

To predict the species composition of a sample, 454 pyrosequencing fragments that had a Pfam hit were labeled according to the longest common prefix shared by the most similar members of the Pfam family. The strategy for assignment was as follows: First, the sequence was compared to each member of the Pfam family with a pair-wise alignment using BLAST. Second,  $E_{min}$  was assigned as the minimal E-value obtained in the BLAST comparisons. The taxa of the organisms of all Pfam family members that hit the sequence with an E-value  $< E_{min} + 5\% \cdot E_{min}$  were fetched from NCBI. Finally, the sequence was labeled with the longest common prefix of these taxa. For example, *Bacteria:Cyanobacteria* is the longest common prefix of the two taxa

- *Bacteria:Cyanobacteria:Prochlorales:Prochlorococcaceae:Prochlorococcus*
- *Bacteria:Cyanobacteria:Chroococcales:Synechococcus*

The taxonomic identification provided by this analysis was categorized according to the definitions in Bergey's manual, thus enabling a comparison of the metagenomic data using a separate database.

### Vibrio spp. Culturing

Bacteria were cultured using thiosulfate citrate bile sucrose (TCBS; Remel; Lenexa, KS USA) plates to provide an estimate of the number of culturable *Vibrio* spp. present on each atoll. Water was sub-sampled from the Niskin bottles and 0.1 ml was inoculated on eight plates for each site. To determine the numbers of *Vibrio* spp. associated with coral, mucus samples were obtained from six corals per site using specially developed "super-suckers". This apparatus allows the mucus to be gathered from the coral colony while minimizing the entry of surrounding seawater. Once again, 0.1 ml of coral mucus was spread onto plates. *Vibrio* spp. colonies forming units were enumerated after 24 hours incubation at 28°C (ambient sea water temperature). Differences in *Vibrio* spp. concentrations per ml of seawater or mucus were compared across all four atolls simultaneously using a non-parametric ANOVA. To further assess differences in the number of culturable *Vibrio* spp. between atolls, pairwise comparisons for all possible pairs of atolls were conducted using non-parametric exact Wilcoxon tests, testing a one-sided alternative hypothesis (i.e., that one atoll in the pair had a higher concentration of *Vibrio* spp. than the other). The one-sided test was conducted for all atoll pairings. On Kingman no culturable *Vibrio* spp. were observed in either the seawater or coral mucus, so the TCBS plates were tested with isolates from Kiritimati. In all cases, the plates were able to support growth of *Vibrio* spp.

### Water chemistry

Dissolved organic carbon (DOC), total dissolved inorganic nitrogen (nitrate, nitrite and ammonium), and dissolved inorganic phosphate were determined for all Niskin bottles. For DOC analysis, the water samples were filtered through a pre-combusted Whatman GF/F glass fiber filter and collected in pre-combusted amber glass vials (Wheaton) with acid-washed Teflon lined lids. Samples were acidified (~pH 2) with analytical grade 30% hydrochloric acid (Fluka) and stored at 4°C. DOC concentrations were analyzed by Expert Chemical Analysis (San Diego, CA) using the high-temperature combustion method and a O.I. Analytical Model 1010 TOC analyzer (Texas, USA). To ensure quality control, DOC consensus reference materials (CRM: DSW Lot 05-05 at 45-46 µM C; LCW Lot 12-01 at 2 µM C; supplied by Dr Wenhao Chen, University of Miami) were used, and the high-carbon standard was run every six samples. The methods described for the DOC analysis are the same as those prescribed by the Intergovernmental Oceanographic Commission for the collection of DOC samples. These protocols avoid many of the uncertainties associated with earlier research on DOC levels [45,46].

For inorganic nutrient analysis, samples were filtered through 0.2 µm Nuclepore Track-Etch membrane filters (Whatman) into HDPE scintillation vials with cone-shaped plastic lined lids (Fisher Scientific), after rinsing both the bottles and lids three times with filtrate. Each sample consisted of 15ml of filtrate, which were then stored at -20°C until analyzed. Analysis of inorganic nutrient (nitrate, nitrite, ammonium and phosphate) concentrations was carried out by Marine Science Institute Analytical Lab at University of California at Santa Barbara (Santa Barbara, CA) using a QuikChem 8000 flow injection analyzer (Lachat Instruments,

Wisconsin, USA). All chemical components were found not to vary between depths (DOC  $F_{32} = 0.872$ ,  $P = 0.660$ ; TDIN  $F_{32} = 0.968$ ,  $P = 0.572$ ; Phosphate  $F_{32} = 1.048$ ,  $P = 0.442$ ), and therefore, analyses were conducted on the site level. All water chemistry variables were normally distributed and did not show heterogeneity (tested using test described earlier). The differences between the concentrations of each chemical component on each atoll were compared using a MANOVA with sites nested within locations.

To determine natural isotopes of nitrogen, 1 liter of seawater was filtered through GF/F filters to collect particulate organic matter. The filters were folded in half, wrapped in foil, and stored at -20°C. The  $^{14}\text{N}/^{15}\text{N}$  ratios were determined at the Marine Sciences Institute at UCSB (Santa Barbara, CA) using a Finnigan Delta Plus Advantage with a Costech Elemental Analyzer peripheral. Measurements conducted at Kingman and Kiritimati only.

### Respiration experiments

Micro-respiration experiments were used to estimate the "nutritional quality" of the waters from the four atolls. On each atoll, water collected from three sites only, was 0.2 µm filtered to remove microbes, and stored frozen at -80°C. Upon return to San Diego State University, the samples were thawed and inoculated with the same microbial community to determine whether the waters from one atoll would support more or less microbial activity. The microbial community was obtained by centrifuging 1 ml of water from a laboratory coral reef tank at ~5,000 ×g in a microfuge. The supernatant was aspirated away, and the microbial pellet was resuspended into 1 ml of the previously thawed water from the different atolls (each sample run in triplicate). The mixture of the aquarium microbes and atoll water was placed in micro-respirometry chambers and the oxygen concentrations were measured using micro-oxygen sensor probes (Unisense, Aarhus C, Denmark). The microbes were grown for a total of four hours and oxygen concentrations (nmol O<sub>2</sub> ml<sup>-1</sup>) were measured every minute. Negative controls were conducted by running the experiments without microbial inoculations. To calculate respiration rates, the slopes of the oxygen concentration curve between 75 to 175 minutes were determined. The time-frame was used because it produced the most consistent oxygen utilization levels (i.e., a linear slope after the lag phase). The number of cells in each chamber was determined by direct counts (described above) at the end of the experiment and the respiration rate per million microbes was calculated by dividing by the number of microbial cells. Conventional methods of measuring growth rates of microbes (e.g., using  $^3\text{[H]}$ -thymidine) were not performed because there was no access to a "rad van" on the cruise. Measurements of microbial activity using Br-dUTP incorporation were attempted, but failed because of technical problems. In the end, however, measuring the oxygen consumption by the same microbial community in the different water samples was probably more illustrative than comparing growth rates of different communities in different waters.

### Coral health

Surveys describing the health status of corals were conducted on two 2 m × 20 m belt transects at 10–12 sites on each of the four atolls. These techniques for the assessment of coral health were developed by the Coral Disease working group of the Global Environmental Facility/World Bank coral reef targeted research program [14]. All coral colonies, both healthy and those showing signs of potential disease were counted. Most coral diseases do not have known microbial pathogens, but the colony displays signs that can be distinguished. These signs included White Syndrome, Skeletal Eroding Band, Brown Band, Black Band and other

cyanobacterial infections [47], tissue necrosis caused by sediments containing a high mucus load and low numbers of cyanobacteria, algal interactions with corals that cause tissue erosion, bleached white patches, and pink coloration [48,49]. Predator feeding scars from *Drupella* spp. and *Acanthaster planci* were identified and excluded from the analysis. Corals showing signs of compromised health were used to analyze the relationship between the number of unhealthy and healthy corals at each site. The prevalence of unhealthy corals was calculated by dividing the numbers of unhealthy corals by the total number of corals on each transect. Only scleractinian corals were used in the assessment.

### Percent cover of benthic organisms

Quantitative assessments of the benthos were made using the photoquadrat method [50]. Ten points were randomly selected and surveyed per transect (i.e., the same transects that were laid for the coral health surveys). At each point a photograph was taken using an Olympus 7070 digital camera that was connected to a tripod (1 m high) and a frame (0.9×0.6 m or 0.54 m<sup>2</sup>). Therefore, for each site 20 photoquadrats were sampled. During surveys, notes were made for each photoquadrat and collections were made for organisms that were unidentifiable *in situ*. Upon return to the research vessel, the twenty photographs were numbered and color adjusted using Adobe Photoshop v. 7.0. Image analysis was completed using the program Photogrid 1.0. For each photograph, 100 points were randomly generated and the organism under each point was identified. Therefore, for each site 2000 points were quantified. All organisms were identified to the finest level of resolution possible (genus level for scleractinian and soft corals, functional group for algal turfs and crustose coralline algae, and species level for macroalgae and macroinvertebrates where possible). The percent of coral cover was calculated by dividing the number of points that were assigned to scleractinian corals by the total number of points counted for each photoquadrat. Percent cover of scleractinian corals was used to identify the density dependent nature of the distribution of unhealthy corals on each atoll.

## Results

### Microbial and viral abundances

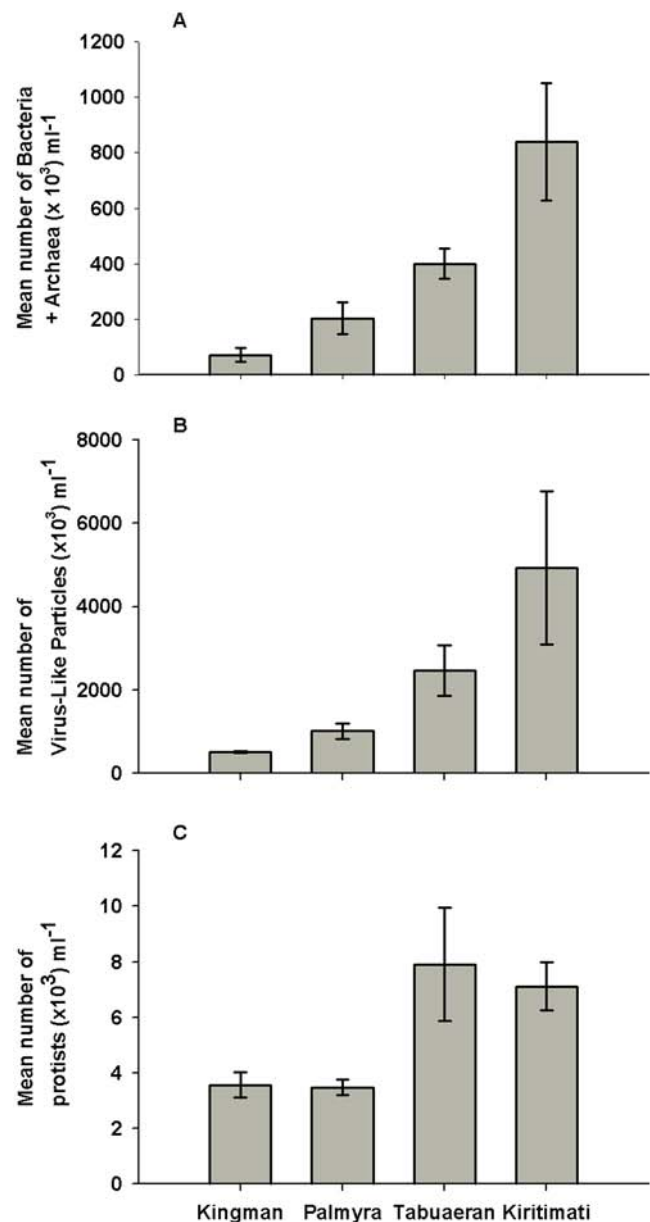
The microbial abundances (Bacteria and Archaea) and virus-like particles (VLPs) both increased by about an order of magnitude from Kingman to Kiritimati (Figure 2A and B;  $F_{3, 12} = 11.5$ ,  $P < 0.001$  for microbes;  $F_{3, 12} = 10.5$ ,  $P < 0.001$  for VLPs). In both cases, the increase was progressive from Kingman ( $7.2 \times 10^4 \pm 1.7 \times 10^4$  microbes ml<sup>-1</sup> and  $5.1 \times 10^5 \pm 1.5 \times 10^5$  VLPs ml<sup>-1</sup>) to Palmyra ( $2.0 \times 10^5 \pm 1.7 \times 10^4$  microbes ml<sup>-1</sup> and  $1.0 \times 10^6 \pm 3.7 \times 10^5$  VLPs ml<sup>-1</sup>) to Tabuaeran ( $4.0 \times 10^5 \pm 2.3 \times 10^4$  microbes ml<sup>-1</sup> and  $2.5 \times 10^6 \pm 1.2 \times 10^6$  VLPs ml<sup>-1</sup>) to Kiritimati ( $8.4 \times 10^5 \pm 1.4 \times 10^5$  microbes ml<sup>-1</sup> and  $4.9 \times 10^6 \pm 1.1 \times 10^6$  VLPs ml<sup>-1</sup>) (Wilcoxon one-sided pairwise test was significant for all atoll pairings at the  $P = 0.05$  level). Protist abundance doubled from Kingman and Palmyra ( $3575 \pm 457.1$  and  $3486 \pm 275.4$  protists ml<sup>-1</sup>, respectively) to Tabuaeran and Kiritimati ( $7917 \pm 2037.1$  and  $7124 \pm 868.1$  protists ml<sup>-1</sup>, respectively) ( $F_{3, 12} = 15.5$ ,  $P < 0.001$ ; Figure 2C).

### Taxonomical and metabolic potential of microbial metagenomes

Taxonomic analyses of sequences from the large metagenomic fraction showed a non-monotonic change in the relative fractions of autotrophs and heterotrophs on the atolls (Figure 3A). Sequence comparisons with the 16S rDNA database showed that the microbial communities were increasingly autotrophic moving from Kingman (50% of identifiable metagenome sequence were similar

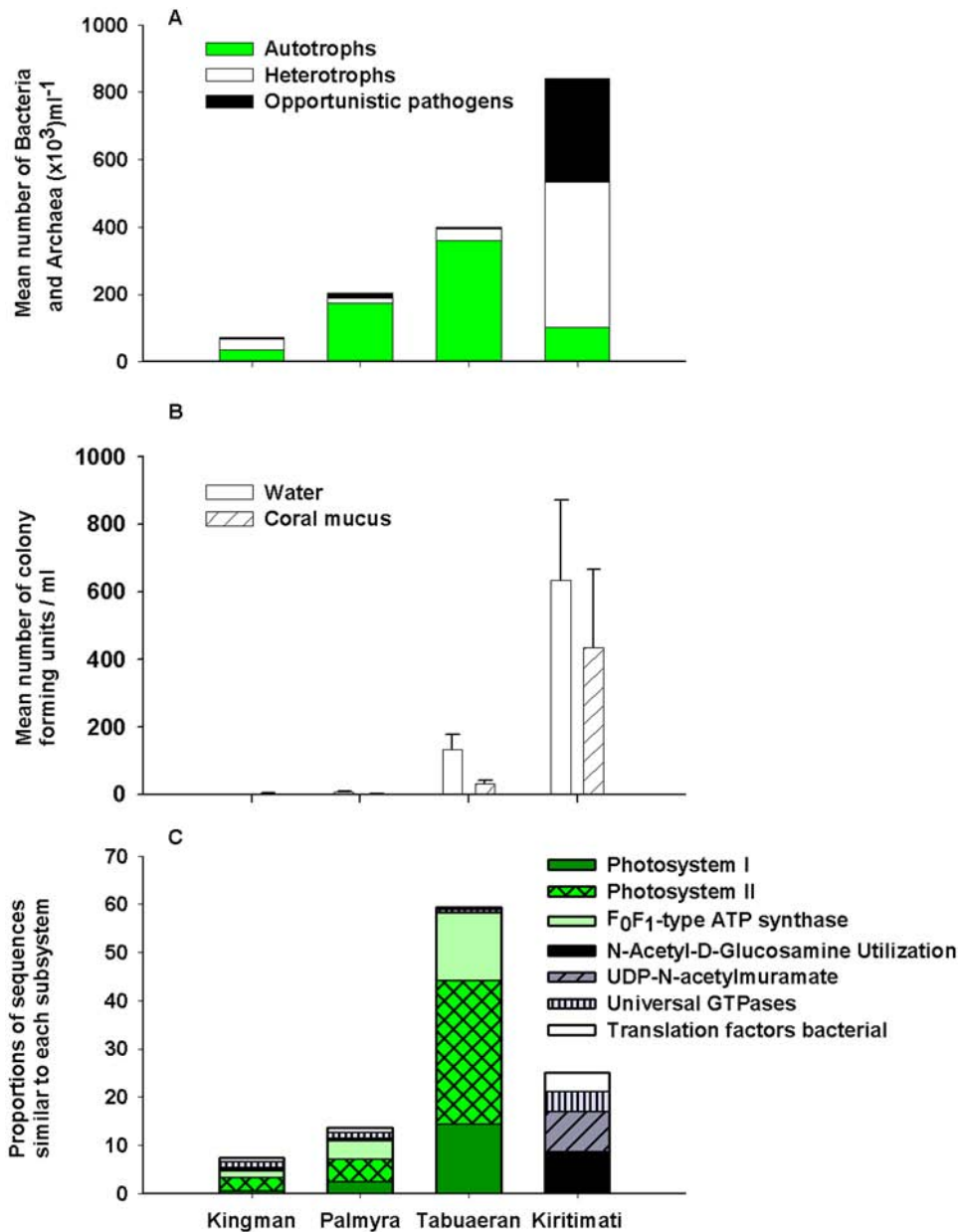
to known autotrophs) to Palmyra (84%) to Tabuaeran (89%), but at Kiritimati the proportion of autotrophs sharply declined to 12% (Figure 3A). The robustness of this trend was supported further by comparisons of the DNA sequences against the SEED platform [36] and the Pfam database [43], which revealed similar changes in the relative proportion of autotrophs across the atolls (Figure S2). Further, the proportion of heterotrophs that were potential pathogens also increased on Kiritimati. The number of culturable *Vibrio* spp. from the water column and coral mucus samples also increased progressively from Kingman to Kiritimati (Figure 3B).

The metabolic potential of the microbial community, determined by comparing the sequences to the SEED platform and categorizing them into metabolic subsystems [36], showed similar patterns. Changes in relative abundance of autotrophic subsystems



**Figure 2. Direct counts were used to determine the mean abundance ( $\pm$  standard error) of A) microbial cells (Bacteria and Archaea), B) virus-like particles (VLPs), and C) protists on the four Northern Line Island atolls.**

doi:10.1371/journal.pone.0001584.g002



**Figure 3. Taxonomic and metabolic potential of Bacteria and Archaea of the four atolls: A) Proportion of autotrophs, heterotrophs and potential pathogens identified by the 16S rDNA sequences in the microbial metagenomic fractions. B) Number of cultured *Vibrio* spp. (bar represents means  $\pm$  standard error) in the water column ( $F_{3,58} = 5.697$ ,  $P = 0.002$ , Wilcoxon one-sided paired t-test showed significant differences for all atoll pairings at  $P = 0.05$ ) and coral mucus ( $F_{3,42} = 3.514$ ,  $P = 0.023$ , Wilcoxon one-sided paired t-test showed significant differences for all atoll pairings at  $P = 0.05$ , except between Kingman and Palmyra  $P = 0.299$ ). C) The metabolic potential expressed by the seven most abundant subsystems, across the atolls. These subsystems varied significantly between Kingman and Kiritimati using both XIPE [37] and G-test (Supplementary data). Subsystems that are more closely associated with autotrophs are shown in green. The "potential pathogen" designation are known human pathogenic genera like *Staphylococcus*, *Vibrio*, and *Escherichia*, fish pathogens like *Aeromonas*, and plant pathogens from the *Xylella* genera. doi:10.1371/journal.pone.0001584.g003**

across atolls paralleled the non-monotonic changes described by the taxonomic analyses (Figure 3C and S3). On Kingman, Palmyra, and Tabuaeran, sequences similar to the Photosystem I and II comprised 3.4, 7.2, and 44.3% of the total identifiable subsystems, respectively, but only 0.3% on Kiritimati (Figure 3C). F<sub>0</sub>F<sub>1</sub>-type ATP synthase, a subsystem that is involved in oxidative phosphorylation, showed a qualitatively similar change as the photosynthetic subsystems; F<sub>0</sub>F<sub>1</sub>-type ATP synthase is often coupled with photosynthesis to produce ATP. The N-Acetyl-D-glucosamine utilization subsystem, which is used in the consump-

tion of fixed carbon and thus associated with heterotrophic growth, was highly represented on Kiritimati (8.2% of the identifiable sequences). In comparison, this subsystem was less than 1% on the other three atolls. Universal Guanosine Triphosphatase (UTPase), Uridine diphosphate-N-acetylmuramate and various translation factors were also highly represented on Kiritimati. Variation in less abundant metabolic subsystems across these atolls is provided in Figure S3.

The types of bacterial autotrophs in the microbial fraction also changed on the atolls. The most common bacterial autotrophic

genus on Kingman and Palmyra was *Prochlorococcus* (75 and 91% of the cyanobacterial population, respectively), whereas on Tabuaeran and Kiritimati, *Synechococcus* was the most common genus (66 and 64% of the cyanobacterial population, respectively; Figure S4). This pattern may reflect variations observed in the water chemistry across the atolls, because *Prochlorococcus* is common in oligotrophic water, whereas *Synechococcus* becomes dominant in increasingly nutrient rich water [51,52].

## Viromes

The viral metagenomic fraction was compared to a database of all known phage and prophage genome sequences (<http://phage.sdsu.edu/phage>). Significant similarities to this database ( $E\text{-value} \leq 0.001$ ) were used to identify the types of phages on each atoll. Since phage are host specific the proportion of phage infecting autotrophic and heterotrophic microbes was calculated. In parallel with the microbial analysis, the analysis of the phage hosts showed the phage known to infect cyanobacteria increased from Kingman (44%) to Palmyra (73%) and Tabuaeran (61%), and then at Kiritimati the phage known to infect heterotrophic microbes became dominant (61%; Figure 4A). A further breakdown of the potential host range of the phage is provided in the Figure S5.

The virome sequences were also analyzed using a fragment recruitment method to known genomes (described in [29]), which maps sequences to their relevant position on the reference genome (Figure 4B). Sequences similar to *Escherichia coli*  $\Phi$  CP4-6 prophage, which is found in highly virulent enterohemorrhagic *Escherichia coli* strains [53], were more common on Kiritimati. In contrast, sequences similar to the *Prochlorococcus marinus* SSMP4 phage were more common in Kingman, Palmyra, and Tabuaeran (Figure 4B). The differences between the sequence distributions also became apparent when the average number of sequences showing similarities to each section of the genome was compared. For example, the number of sequences similar to *Escherichia coli*  $\Phi$  CP4-6 prophage steadily increased from Kingman (29 sequences per 5000 bp), to Palmyra (66 sequences per 5000 bp) to Tabuaeran (91 sequences per 5000 bp) to Kiritimati (147 sequences per 5000 bp).

## Microbial predator-prey ratios

Virus-like particles (VLPs) and microbial numbers were positively correlated on Kingman, Tabuaeran, and Kiritimati, but not on Palmyra (Figure 5). The steepness of the slope of the VLPs:microbes increased from Kingman (0.909) to Tabuaeran (1.378) to Kiritimati (1.768). Microbes on Kiritimati were able to sustain approximately two times the number of VLPs than on Kingman, which suggests that the characteristics of the relationship are not static, but may be associated with conditions on each atoll.

The overall abundances of the protists increased from Kingman to Kiritimati, but the protists:microbe ratio declined. There were 0.0301 ( $\pm 0.018$ ) protists per microbial cell at Kingman, 0.013 ( $\pm 0.005$ ) at Palmyra, 0.015 ( $\pm 0.004$ ) at Tabuaeran, and 0.008 ( $\pm 0.004$ ) at Kiritimati. On Kingman, 66% of protists were strict heterotrophs (i.e., contained no chlorophyll) compared with 22% on Kiritimati.

## Coral cover and disease prevalence

As shown in Figure 6, coral cover declined from Kingman (43.8%  $\pm 5.4$ ) to Palmyra (20.4%  $\pm 2.3$ ) to Tabuaeran (19.5%  $\pm 1.0$ ) to Kiritimati (14.9%  $\pm 2.3$ ), whereas prevalence of disease on hard corals was lowest on Kingman (2.5%  $\pm 0.5$ ) and highest on Kiritimati (6.3%  $\pm 1.4$ ) and Tabuaeran (6.2%  $\pm 1.4$ ). Palmyra showed medium prevalence of disease (4.8%  $\pm 2.0$ ) (Kruskal Wallis test;  $H = 8.0$ ,  $df = 3$ ,  $p = 0.04$ ).

## Water chemistry

Dissolved organic carbon (DOC) concentrations were highest on Palmyra (51.1  $\pm 2.1$   $\mu\text{mol l}^{-1}$ ) and Tabuaeran (49.5  $\pm 2.4$   $\mu\text{mol l}^{-1}$ ), lower on Kingman (42.5  $\pm 0.9$   $\mu\text{mol l}^{-1}$ ), and lowest on Kiritimati (32.3  $\pm 0.6$   $\mu\text{mol l}^{-1}$ ) (Figure 7A). Given the low numbers of measurements of DOC on coral reefs, a range of these values has been provided in Table S2 for comparison.

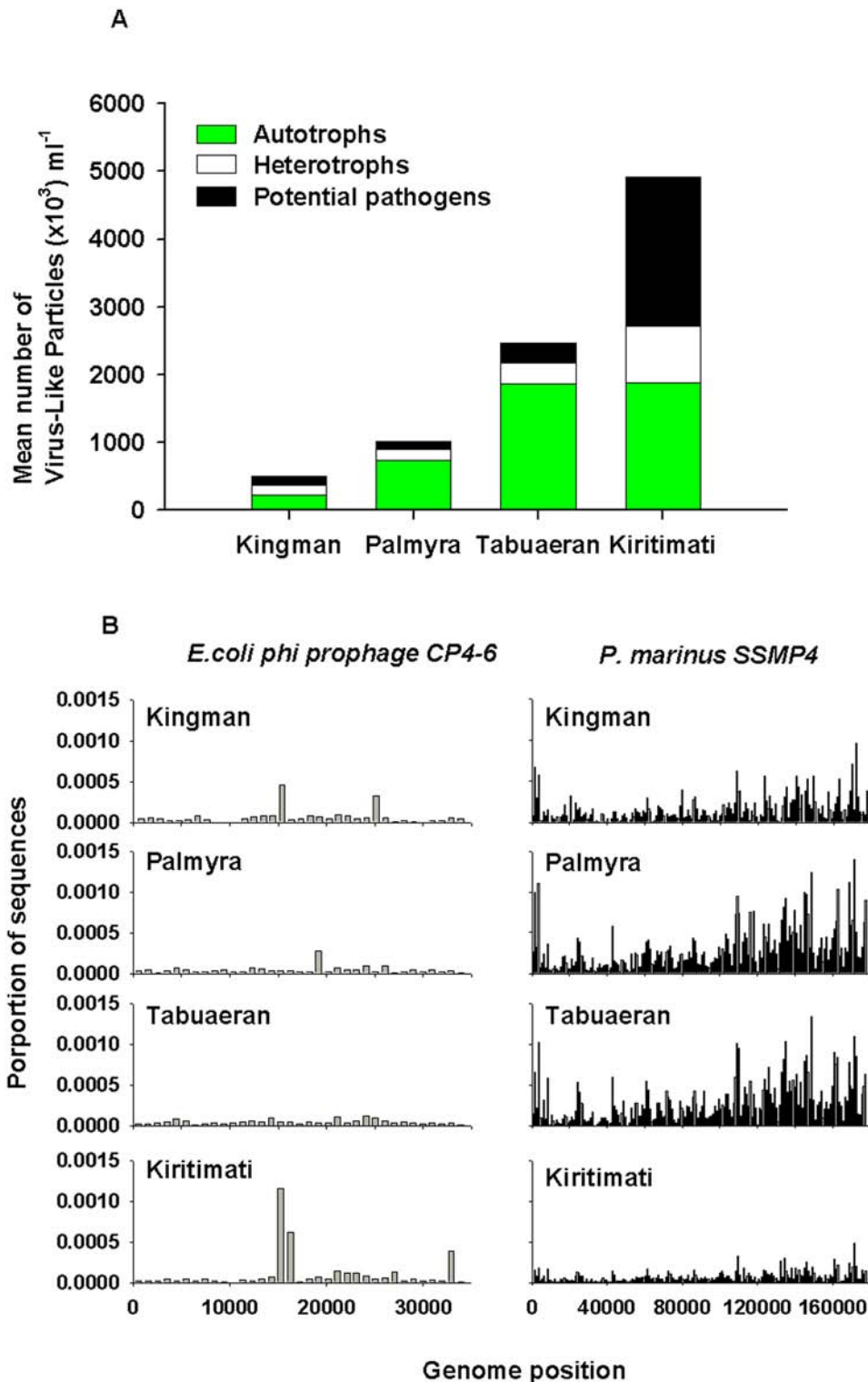
Total dissolved inorganic nitrogen (TDIN) increased almost four-fold from Kingman (1.3  $\pm 0.08$ ) to Kiritimati (3.6  $\pm 0.1$ ) (Figure 7B;  $F_{3,12} = 38.735$ ,  $P < 0.001$ ), and similarly inorganic phosphate concentrations increased from Kingman (0.1  $\pm 0.003$ ) to Kiritimati (0.3  $\pm 0.024$ ) (Figure 7C;  $F_{3,12} = 395.2$ ,  $P < 0.001$ ). No clear pattern was apparent in the concentrations of particulate organic carbon and particulate organic nitrogen (data not shown). No significant differences were found in the  $\delta^{15}\text{N}_{\text{Norm}}$  values in the particulate organic matter from Kingman (4.2  $\pm 0.78$ ) and Kiritimati (5.7  $\pm 1.5$ ) (not measured on Palmyra or Tabuaeran).

These differences in water chemistry were also reflected in results of the assays of oxygen consumption rates of a standard microbial community grown in seawater from each of the atolls. Microbes grown in water from Kingman had the lowest respiration rates (0.058  $\pm 0.012$  nmol oxygen consumed per  $1 \times 10^6$  microbes), whereas the same microbes grown in water from Kiritimati had much higher respiration rates (0.309  $\pm 0.016$  nmol oxygen consumed per  $1 \times 10^6$  microbes) (Figure 7D;  $P < 0.001$ ).

## Discussion

Microbial numbers in the water column overlying coral reefs usually range from  $2\text{--}6 \times 10^5$  cells  $\text{ml}^{-1}$  [2,54,55]. Our mean values were roughly comparable, although the lowest and highest mean values observed exceeded this range: Kingman averaged  $7.2 \times 10^4$  microbes  $\text{ml}^{-1}$ , Palmyra averaged  $2.0 \times 10^5$  microbes  $\text{ml}^{-1}$ , Tabuaeran averaged  $4.0 \times 10^5$  microbes  $\text{ml}^{-1}$ , and Kiritimati averaged  $8.4 \times 10^5$  microbes  $\text{ml}^{-1}$ . Reports for viral like particles (VLPs) range from  $0.3\text{--}1.25 \times 10^7$  VLPs  $\text{ml}^{-1}$  in the water column [56], densities that exceeded those were not observed: Kingman averaged  $5.1 \times 10^5$  VLPs  $\text{ml}^{-1}$ , Palmyra averaged  $1.0 \times 10^6$  VLPs  $\text{ml}^{-1}$ , Tabuaeran averaged  $2.5 \times 10^6$  VLPs  $\text{ml}^{-1}$ , and Kiritimati averaged  $4.9 \times 10^6$  VLPs  $\text{ml}^{-1}$ . For both microbes and VLPs, densities increased steadily across the four atolls; protists also increased, although in a stepwise fashion. There were also differences in community composition, most notably a sharp increase in heterotrophic Bacteria and Archaea and in potential pathogens in Kiritimati. Finally, we observed a steady increase in total dissolved inorganic nitrogen, which was 4-fold higher on Kiritimati than Kingman, and a similar pattern for inorganic phosphate, which increased 3-fold. In contrast, dissolved organic carbon (DOC) concentrations were highest on Palmyra and Tabuaeran and lowest on Kiritimati.

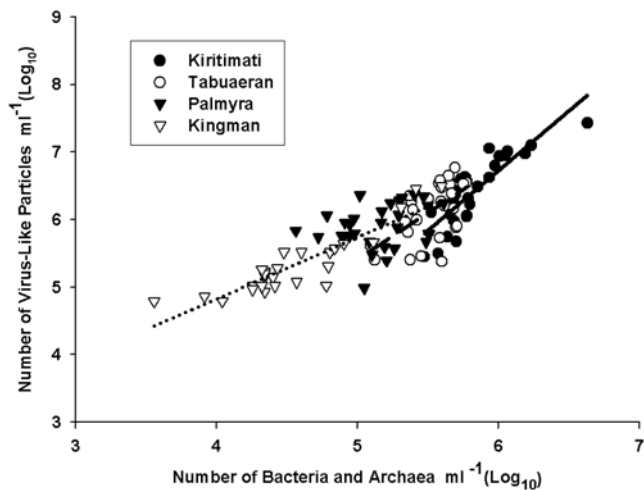
A study of the macrobiota conducted simultaneously with our microbial study documented equally striking changes. Fish biomass dropped steadily from 527 to 132  $\text{g m}^{-1}$  from Kingman to Kiritimati, primarily due to the loss of top predators. In parallel with these differences, coverage of corals and coralline algae declined from 71% to 21%, and cover by fleshy and turf algae increased from  $\sim 20\%$  to 68% from Kingman to Kiritimati [18]. For the macrobiota, historical data and data from nearby Pacific atolls [18] suggest that anthropogenic impacts are likely to be important factors in explaining these differences across the atolls. Historical records for Kiritimati indicate that sharks were once very abundant [57–60], and more recent surveys indicate a decline in fish biomass by 50% and coral cover by 30% in the last decade [61–63]; in contrast, uninhabited Kingman has not suffered such



**Figure 4. Analysis of the viral metagenomes showing: A) The relative abundances of phage host range by guild.** This was the product of the mean number of virus-like particles and the proportion of sequences within the small metagenomic fraction that were similar to autotrophic, heterotrophic or potential pathogenic phage hosts. B) Sequence recruitment across the *Escherichia coli*  $\Phi$  CP4-6 prophage (which is found in highly virulent *E. coli*) and *Prochlorococcus marinus* SSMP4 (which infects an open water autotrophic cyanobacteria). doi:10.1371/journal.pone.0001584.g004

losses. The impact of people on fish communities is uncontroversial. The causes of coral loss are also likely to be anthropogenic, but the relative importance of local impacts (fishing and water

quality) vs. global impacts (especially warming and associated bleaching) is more debated. Also, Jarvis, an uninhabited island with similar oceanographic conditions and global warming conditions



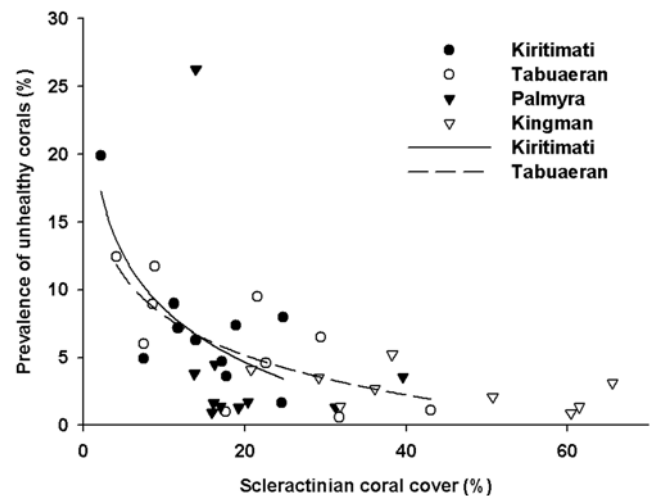
**Figure 5. Relationships recorded between microbes and virus-like particle numbers on the Northern Line Islands.** Kingman (dotted line)  $r^2=0.807$ ,  $P<0.0001$ ; Palmyra  $r^2=0.039$ ,  $P=0.414$ ; Tabuaeran (dashed line)  $r^2=0.324$ ,  $P=0.006$ ; Kiritimati (solid line)  $r^2=0.706$ ,  $P<0.0001$ .

doi:10.1371/journal.pone.0001584.g005

as Kiritimati, resembles Kingman in fish and benthic community structure [61]. One anecdotal consideration that suggests the decline of corals on Kiritimati appears to be a relatively recent event (i.e., within the last decade) is shown in seascape photos in Figure 8. Large coral skeletons were still free-standing in the algal-dominated reef areas and many of the still-living coral colonies were relatively large, with high levels of partial mortality (circles in photoquadrates). Similarly, surveys conducted in 1997 for a proposed Japanese space site identified higher coral cover on Kiritimati than was recorded in 2007, suggesting that the loss of corals is a recent event [63]. In sum, the historic data and the comparisons with nearby atolls suggest that the benthic and fish communities were originally similar on Kiritimati and Kingman in recent time [18].

However, for the microbes, there have been no systematic surveys on these atolls, including Jarvis, so interpreting the patterns observed is more complex. Microbial communities respond to the characteristics of seawater, which are affected by regional oceanographic differences, including local upwelling, lagoon influences, land run-off, and the benthic community structure (especially the amount of benthic algae). The last three of these can be affected by both the physical and oceanographic characteristics of the atolls, by the activities of people locally, and anthropogenic global change. There are thus two competing, but not mutually exclusive, hypotheses to explain the observed microbial and macrobiota patterns in the Northern Line Islands (outlined in Table 1). Since the four atolls are different sizes and are separated by ~750 kilometers along a north-south transect, regional differences and/or reef hydrology may be the primary driving factors for differences in the measured parameters. Alternatively, the varying levels of human disturbance associated with sewage (there is little industry or large-scale agriculture) and fishing that the atolls experience, or varying amounts of temperature stress associated with global warming may account for the observed differences.

Some of the differences among the atolls are probably long-standing and reflect oceanographic and hydrographic differences, with predictable consequences for microbial community composition. Moving south from Kingman, the atolls are progressively larger with consequently greater potential to induce upwelling, larger

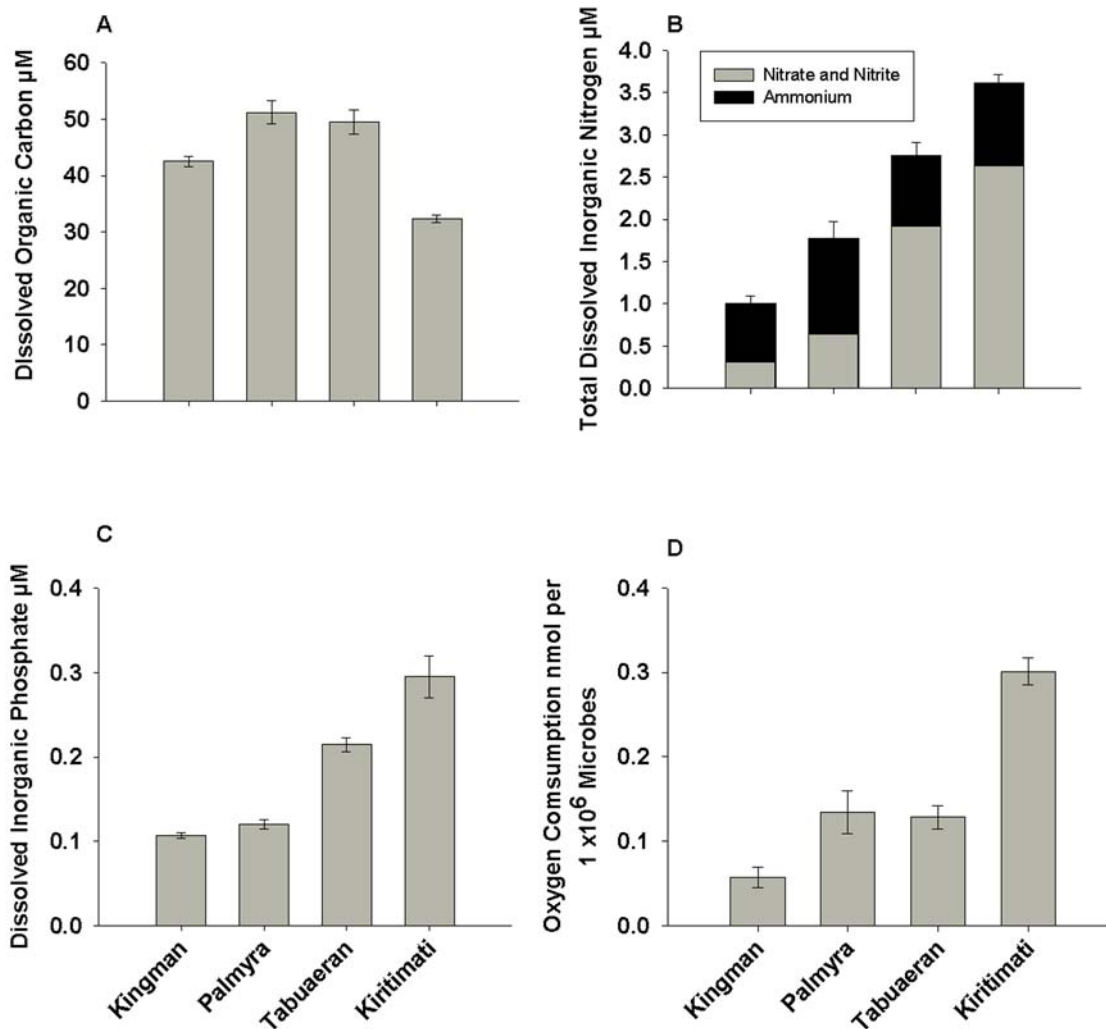


**Figure 6. Prevalence of unhealthy scleractinian corals compared with scleractinian coral cover.** The prevalence of unhealthy corals was negatively related to host density on both Tabuaeran ( $r^2=0.477$ ,  $P=0.002$ ) and Kiritimati ( $r^2=0.664$ ,  $P=0.003$ ). No relationship was found on Palmyra ( $r^2=0.261$ ,  $P=0.141$ ) or Kingman ( $r^2=0.251$ ,  $P=0.300$ ).

doi:10.1371/journal.pone.0001584.g006

lagoons, and larger seabird populations. All of these trends could influence microbial communities. For example, on Kingman, the autotrophic and heterotrophic microbial communities in the water column were roughly balanced. In oligotrophic waters, photosynthetic cyanobacteria are the major energy producers [64] and compete with the heterotrophic bacterial communities for inorganic nutrients [52]. *Prochlorococcus* utilizes reduced forms of nitrogen and loses competitive dominance in seawater where the levels of nitrates are high [64,65]. On Tabuaeran, the photosynthetic microbes made up 80% of the community and photosynthetic subsystems comprised over 40% of the sequences identified within the metagenome. The dominance by *Synechococcus* correlated with the increase in nitrogen and phosphate concentrations in the water and is similar to the large scale distribution patterns of autotrophs in the ocean [64]. The increase in photosynthesis on Palmyra and Tabuaeran may have caused the increased concentration of DOC on these atolls (Figure 7A). Similarly, the metagenomes showed that the number of microbial autotrophs in the 0.45–100 micron fraction increased from Kingman (50%) to Palmyra (84%) to Tabuaeran (89%) (Figs. 3A and C). This trend correlated well with the increasing concentration of fixed nitrogen compounds (nitrate, nitrite, and ammonium) and phosphate in the water column (Figures. 7B and C) and may be due to increased upwelling on the progressively larger atolls. The concentrations of nitrate/nitrite and phosphate continue to increase on Kiritimati, but the microbial community became predominantly heterotrophic in nature (72%), suggesting an available carbon source. This observation is consistent with the hypothesis that nutrients from upwelling, and possibly runoff from the island, combined with a loss of herbivory are stimulating benthic macroalgae and phytoplankton. In turn, the algae produces dissolved organic carbon (DOC) which supports more heterotrophic bacterial growth. This is additionally supported by the observation that both the algal cover and the highest numbers of microbes were also observed on Kiritimati.

The apparent inconsistency with the hypothesis that high levels of DOC released by algae are increasing heterotrophic bacterial is that the lowest DOC concentrations were observed on Kiritimati. Similar phenomena have been observed on other coral reefs in the Caribbean and Sri Lanka (strong correlation between higher



**Figure 7. Water chemistry measured for the four Northern Line Island atolls.** Concentrations of A) Dissolved organic carbon (DOC), B) Total dissolved inorganic nitrogen (TDIN: nitrite and nitrate, and ammonium), and C) Dissolved inorganic phosphate are presented as means ( $\pm$  standard error). D) Microbial respiration rates as determined by adding the same microbial communities to samples of seawater collected from the four atolls. doi:10.1371/journal.pone.0001584.g007

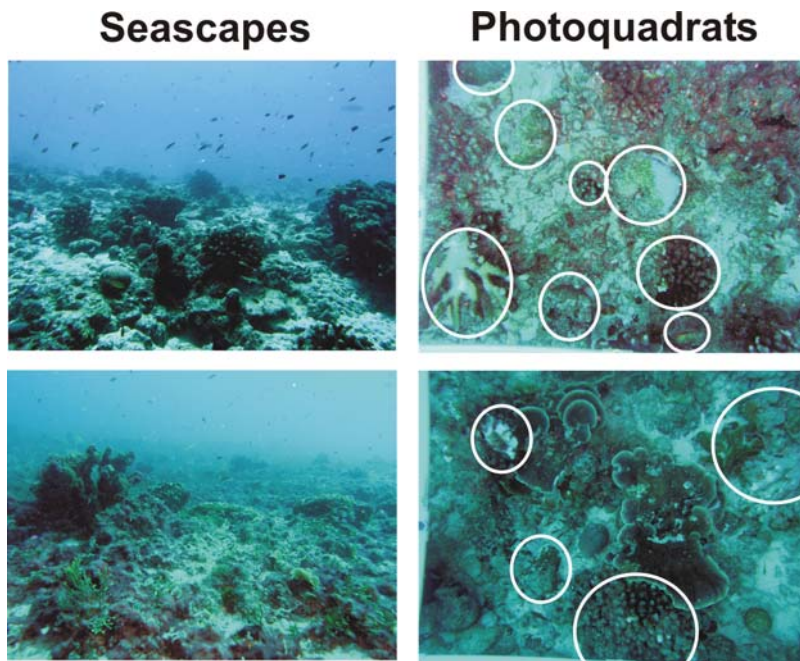
microbial numbers and lower DOC; Pantos, Fairouz, Rohwer; unpublished data). While this may seem counter-intuitive, the lower DOC concentrations are the result of co-metabolism of refractory carbon sources that occurs when microbes are given an excess labile carbon. Carlson et al. [66] showed that increases in inorganic nutrients alone were insufficient to enable bacterial communities to utilize refractory DOC, but required an addition of a bio-available source of DOC. Further, when the labile carbon was supplied, the taxonomic composition of the microbial communities changed (the study by Carlson et al, however, did not identify the microbes) [66], similar to the differences in taxonomic composition that were observed across the four coral atolls. Similarly, fresh carbon supplied to soil microbes enabled the mineralization of old carbon [67]. Consistent with this explanation, addition of the same laboratory microbial community to seawater samples from all four coral atolls showed that the lower DOC-containing water from Kiritimati supported more microbial respiration (Figure 7D).

Total nitrogen inputs associated with sewage were estimated to be 227 and 397 kg N<sup>-1</sup> km<sup>-1</sup> yr<sup>-1</sup> for the inhabited coastline of Tabuaeran and Kiritimati, respectively [18]. Given the large volumes of water that moves passed these reefs, we expect that this extra nitrogen from sewage will be diluted out. While these

nutrients may have influenced the microbial community to some extent, they are a fraction of the inputs estimated on highly populated reefs, such as Florida Bay [68]. Additionally, no evidence of human sewage was apparent in the isotopic signature of the particulate organic matter  $\delta^{15}\text{N}_{\text{Norm}}$  values from Kiritimati ( $5.7 \pm 1.5$ ) compared with Kingman values ( $4.2 \pm 0.78$ ). Therefore, human-derived sewage does not seem to be the reason for the elevated nutrients on Tabuaeran or Kiritimati. Bird guano, however, is a potential influence that was not controlled for in this study and may explain some of the elevated nutrient concentrations on Kiritimati, Tabuaeran, and Palmyra.

Increasing atoll size and oceanographically more oligotrophic water were directly correlated with significant increases in protists, microbes, and VLPs. However, the decreasing percentage of heterotrophs from Kingman to Palmyra, followed by an abrupt shift to a heterotroph dominated-community on Kiritimati, does not directly match this pattern. The most straight-forward explanation, as presented above, is that an increase macroalgae, and possibly phytoplankton, is producing labile DOC that supports the change in the microbial community on Kiritimati.

Disease incidence on coral reefs are associated with human activities [69,70]. Changes in the chemical composition of



**Figure 8. Seascape and photoquadrat photographs obtained from the metagenomic sampling site on Kiritimati. White circles indicate diseased, bleached, or recently dead corals.**  
doi:10.1371/journal.pone.0001584.g008

seawater may affect coral disease levels, either by favoring the growth of pathogens and/or decreasing the resistance of the coral animal to infection. Increases in inorganic nutrients are typical on coral reefs influenced by human activities and have been implicated in increasing severity of fungal infections of corals [71]. However, recent experiments suggest that dissolved organic carbon (DOC) may also be important. Experimental dosing of coral fragments with increased inorganic nutrients did not increase coral mortality, but the addition of DOC caused tissue necrosis and mortality [72,73] and increased microbial growth. Another common coral stressor, sedimentation, also causes coral tissue loss and mortality in the presence of high organic material [74,75]. Treatment of organic laden sediments with antibiotic stopped the coral mortality [76]. Smith et al. [77] showed that corals died when placed adjacent to macroalgae, even when separated by a 0.02  $\mu\text{m}$  membrane that was impermeable to viruses and microbes, but not dissolved compounds like DOC. The algae increased microbial growth on the coral, which in turn caused hypoxia and presumably the coral mortality. Coral mortality did not occur in this experiment when antibiotics were added [77]. These results suggest that algal-derived DOC may be a primary driver of coral-microbial interactions. In addition, algae-associated microbial communities harbor pathogens that cause coral disease [78].

Potential pathogens were proportionately more abundant in the Kiritimati microbial metagenomic sample (36.3%; Figure 3A), including many bacterial genera and species that are known pathogens of eukaryotes (Figures. 3A 3C and S5) and human pathogens like *Staphylococcus*, *Vibrio*, and *Escherichia*. The culturable *Vibrio* spp. data support this observation (Figure 3B), as do the metagenomic analyses of the viromes (Figures 4 and S5). While it is not possible to absolutely prove (because of microbial genomic plasticity) that these cultured and uncultured data represent pathogens, the combined data is indicative of unhealthy waters. The increase in potential pathogens could be caused by changes in DOC, which stimulates heterotrophic microbial growth or by increased input of pathogens from the humans and animals living

on Kiritimati. The human introduction of pathogens suggested for *Serratia* spp. infection of acroporid corals in the Florida Keys [79], but may be less likely on Kiritimati given the lack of sewage signature.

Whatever the source, increases in potential pathogens may contribute to the documented recent loss of corals and present patterns of prevalence of disease. Doubling the concentration of culturable *Vibrio* spp. or enteric-like microbes in the water column caused 100% coral mortality under experimental conditions [72]. Therefore, the observed ten-fold increase in abundance of microbes, in both the direct counts and by culturing, has the very real potential of killing corals in Kiritimati.

The hypothesis that the Kiritimati microbial community is detrimental to corals raises the important question: Is this type of microbial community something that should be expected on coral reefs? The sampling scheme used in this study did not find regions of high heterotrophic activity on Kingman, Palmyra, or Tabuaeran. The sampling was performed at defined distance intervals, which resulted in a more complete survey of the smaller islands. However, a possibility remains that we failed to find the right area on the other atolls that had the higher microbial communities. Regional differences are also a possible explanation for the observed data. Kiritimati may have bleached in the relatively recent past (a good candidate is the 1998 warming event) [18]. If this event killed the corals, then algae could have colonized the area. In this case the microbial mechanisms discussed above could help prevent recolonization by corals.

The hypothesis we favor, however, is that a change in the food web structure explains the observed differences. On Kingman and Palmyra, there was no significant relationship between disease prevalence and host density, whereas disease prevalence was negatively related to host density on Tabuaeran and Kiritimati (Figure 6). Generally, a density dependent relationship exists between the hosts and pathogens, with the prevalence of disease increasing with host density [80]. The loss of the density dependent nature of the host-pathogen relationship suggests

**Table 1.** Summary of observations and possible interpretations of microbial and macro-organism data collected from the Northern Line Island survey.

Change as observer moves from Kingman to Kiritimati	Interpretation for hydrology/regional hypothesis	Interpretation for Human-driven food web shift hypothesis
Increased nutrients	a) Upwelling	a) Upwelling
	b) Terrestrial runoff (guano, sewage, agriculture, vegetation)	b) Terrestrial runoff
		c) Increase nitrogen fixation by cyanobacteria/turf algae*
Increased # of microbes and viruses	More microbes come from the larger lagoons	a) Overfishing of herbivores leads to more labile DOC
	Why do the herbivores not graze down the new algae?	b) Increased nutrients lead to more photosynthesis and DOC for microbes
Change from autotrophic to heterotrophic microbial communities	???	More labile DOC to support heterotrophs from unchecked macroalgae growth
More culturable <i>Vibrio</i> spp. and pathogen-like heterotrophs	???	Shift in types of <i>Vibrio</i> spp. due to DOC lability
<i>Prochlorococcus</i> to <i>Synechococcus</i> & autotrophic protists	Increased nutrients due to upwelling	Increased nutrients due to upwelling
Decreased coral cover	a) There were never corals in surveyed regions of Kiritimati	a) Overfishing increases labile DOC, increased coral-algal interaction zones, & pathogen reservoirs
	b) Coral bleaching killed corals	b) Coral bleaching leads to increased disease incidences
	Why do the Kiritimati corals look recently dead?	
Increased algal cover and shift from coralline to fleshy/turf algae	Nutrients favor fleshy and turf algae	a) Overfishing leads to decreased grazing
		b) Nutrients favor fleshy and turf algae
Increased coral disease	???	More pathogen-like microbes = more disease
Non-linear change in coral cover/disease prevalence	???	Same as above
Lower coral recruitment	Algae occupy substratum	a) Pathogens kill off recruits
		b) Algae occupy substratum
Losses of top predators in historical records	Bleaching destroys structure and fish leave	Overfishing
Inverted food pyramids for fish	Same as above	Same as above

Two hypotheses are considered: **Hydrology/regional hypothesis**-Larger islands are associated with more upwelling and algae. Different levels of bleaching on the various islands are the explanation of historical changes (i.e., loss of corals cause fish to leave). For example, the lagoons of Kiritimati and Tabuaeran may have served as sources of hot water during a warming period in the region. **Human-driven food web shift hypothesis**-Overfishing increases macroalgae, which increases amount of labile dissolved organic carbon (DOC). In turn the DOC increases heterotrophic/pathogenic microbes, which kill corals. These two hypotheses are not mutually exclusive. For example, algal-microbial dominated system may represent an alternate stable state initiated by a bleaching event. Where multiple interpretations are given, they are ranked in order of possible importance. Some questions for consideration are highlighted in red.

\*Decreased grazing leads to higher concentrations of turf algae [18,94]. These turfs contain cyanobacteria that fix nitrogen.  
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environmental factors are increasing opportunistic coral diseases. The proposed mechanism is that overfishing removes both predatory and herbivorous fish. Loss of the herbivorous fish results in more algae and microbial growth, which leads to an increased coral death via the microbial mechanisms described above. Removal of the top predators (i.e., top down control) slows down the rate at which energy turns over in the system. This extra energy, in the form of DOC, supports more heterotrophic microbes. Obviously, this is a complex feedback between fish, algae, microbes and coral health that requires further investigation.

#### Future studies to differentiate between regional/hydrological and food web hypotheses

Table 1 outlines a number of observations and their interpretation in the context of the two competing hypotheses. The main differences revolve around the ultimate cause of coral reef decline. Global and regional phenomena are the major factors structuring coral reefs and their geotemporal rise and decline. The current global decline in coral reefs, however, is almost certainly human-driven. Coral bleaching, caused by rising sea surface

temperatures, can devastate coral reefs. Microbes are assuredly important components of this stress, either as primary causes [81–84] or as opportunistic pathogens that kill the weakened corals. Bleaching and other perturbations that destroy the structure of the reef appear to drive coral reefs into another stable state and yield observations similar to what was observed on Kiritimati. Cores will be able to determine if the areas outside of the lagoon have always had low coral cover, or if this is a relatively recent event as suggested by Figure 8. A complete survey of Kiritimati will be able to determine if the rest of the atoll (which includes areas that are not fished or adjacent to villages) has lost its coral cover and subsequent fish populations. If the coral communities are still in place, this would argue against a large scale bleaching event as the triggering event. One caveat is that local hydrology could protect one part of the island, while another area bleaches. Again, cores should help differentiate between these possibilities. Surveys of additional coral reefs would help establish whether there are correlations between coral condition and changes in the microbial communities. The most straight-forward study to test the hypothesis that microbial numbers are driven by increased

macroalgae growth and release of DOC, would be a caging experiment where grazers are added back to a degraded reef to determine if the microbial communities respond. While many caging experiments have been conducted (normally excluding herbivores), none have measured DOC and microbial numbers. Obviously understanding coral reef decline is an active area of research, and the survey presented here provides some insights into microbial involvement in that process. It is important to establish the mechanism driving changes in microbial growth and coral condition because of their importance for management actions.

## Conclusions

In the last thirty years, coral reefs worldwide have suffered an unprecedented loss of coral cover [85]. The positive correlation between human-associated disturbance and coral reef decline is now clear, but there is considerable debate about the precise mechanisms of coral loss. Research to identify these mechanisms has focused on the effects of overfishing, habitat destruction, tourism, global warming, and increases in nutrients from terrestrial run-off [86–88]. With the exception of direct destruction (cyanide, blasting, construction), it is not clear why corals actually die. Bleaching, while important does not always lead to coral mortality [89], direct overgrowth by algae is insufficient to explain the widespread loss of corals. An obvious common denominator in the major scenarios of coral death is disease caused by microbes, either as epidemics caused by specific microbes, such as white band disease which devastated acroporid corals in the Caribbean [90] or opportunistic pathogens as suggested on Kiritimati and Tabuaeran. Specific pathogens can also cause food web shifts, such as the phase shift triggered by the disease of the sea urchin *Diadema* spp. in the Caribbean [91–93]. As in the overfishing food web shift proposed above, opportunistic pathogens were probably the ultimate cause of coral death after the sea urchin die-off. Ecosystem-based management of coral reefs has traditionally focused on animals and plants. Our findings highlight the need to explicitly include microbial processes and their influence on coral reef ecosystem function. Such a framework is also needed to elucidate factors that sustain coral health.

## Supporting Information

**Figure S1** Underwater sampling equipment used to obtain the 150-liter water sample for the metagenomic analysis. The water was taken from the surfaces and crevices of the reef structure. Found at: doi:10.1371/journal.pone.0001584.s001 (1.01 MB TIF)

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**Figure S2** The taxonomic components of the large metagenomic fraction analyzed via sequence similarities to the A) whole genome within the SEED platform, and B) Pfam database. Found at: doi:10.1371/journal.pone.0001584.s002 (2.18 MB TIF)

**Figure S3** The subsystems that showed differences between Kingman and Kiritimati. Found at: doi:10.1371/journal.pone.0001584.s003 (2.18 MB TIF)

**Figure S4** Proportions of *Prochlorococcus* and *Synechococcus* present in the large metagenomic fraction. Found at: doi:10.1371/journal.pone.0001584.s004 (2.11 MB TIF)

**Figure S5** The percentage of the predicted host range of phage in the small metagenomic libraries. Found at: doi:10.1371/journal.pone.0001584.s005 (2.18 MB DOC)

**Table S1** Total number of sequences retrieved in each metagenomic library and the number that showed similarities to those stored in the SEED platform. Found at: doi:10.1371/journal.pone.0001584.s006 (0.03 MB DOC)

**Table S2** Nutrient and organic carbon concentrations measured on coral reefs. Found at: doi:10.1371/journal.pone.0001584.s007 (0.10 MB DOC)

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All metagenomic data is held at <http://scums.sdsu.edu.au>, an open access website. Data are being released through SEED platform (<http://www.theseed.org>) and GenBank short read database. SEED accession numbers: Kingman microbial-4440037.3, Palmyra microbial-4440039.3, Tabuaeran microbial-4440279.3, Kiritimati microbial-4440041.3, Kingman viral-4440036.3, Palmyra viral-4440040.3, Tabuaeran viral-4440280.3 and Kiritimati viral-4440038.3. Genbank accession numbers: Kingman microbial-28343, Palmyra microbial-28363, Tabuaeran microbial-28367, Kiritimati microbial-28347, Kingman viral-28345, Palmyra viral-28365, Tabuaeran viral-28369 and Kiritimati viral-28349. Direct access to data from these metagenomes is available at <http://www.theseed.org/DinsdaleSupplementalMaterial/>.

## Author Contributions

Conceived and designed the experiments: FR ED OP ES SS. Performed the experiments: SS MH FR OP RE EB RV MH. Analyzed the data: ED RE FA DH LK. Contributed reagents/materials/analysis tools: FR LW. Wrote the paper: FR ED. Other: Expedition organizer: SS. Commented on paper: NK. Contributed to design: FA. Supported field work for ED: BW. Organized expedition: ES.

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