**Chagos coral bacteria**

Geoffrey L. Zahn1, AndersonMayfield2, Geoff Students, Danwei Huang3,4, Benjamin J. Wainwright5

1Biology Department, Utah Valley University, 800 W. University Parkway, Orem, UT, 84058, USA.

2Anderson address

3Department of Biological Sciences, National University of Singapore, 16 Science Drive 4,

Singapore 117558, Singapore.

4Tropical Marine Science Institute, National University of Singapore, 18 Kent Ridge Road,

Singapore 119227, Singapore.

5Yale-NUS College, National University of Singapore, 16 College Avenue West, Singapore 138527, Singapore

Corresponding Author: Benjamin J. Wainwright, Yale-NUS College, National University of Singapore, 16 College Avenue West, Singapore 138527, Singapore

Email: Ben.Wainwright@Yale-NUS.edu.sg

**Introduction**

Located in the Central Indian Ocean, the Chagos archipelago is one of the most remote coral reef ecosystems on the planet. The archipelago is a British Indian Ocean Territory (BIOT), and was designated a marine reserve in 2010 by the British government. This strictly enforced (De Santo et al. 2011) no-take marine reserve encompasses 640,000 km2 of water, incorporates 70 islands over seven atolls, including the words largest atoll, the Great Chagos Bank (Sheppard et al. 2012; Sheppard et al. 2013; Graham and McClanahan 2013). The previously populated northern atolls have generally hosted only small human populations, estimated never to have exceed more than 2000 inhabitants. In 1973 all inhabitants were removed to make way for a US Military base on the island of Diego Garcia (Gifford and Dunne 2014). To this day, the only inhabitants of the archipelago are military personal and civilian contractors posted to the Diego Garcia base, other than a small recreational fishery around this base, all fishing activities within the 200 mile exclusive economic zone (EEZ) were made illegal in 2010 (De Santo et al. 2011; Ferretti et al. 2018).

The remoteness of the Chagos archipelago and negligible human disturbances have resulted in some of the cleanest and unimpacted seas in the world (Sheppard et al. 2012; MacNeil et al. 2015; Graham et al. 2018; Head et al. 2018), consequently the coral reefs of the archipelago are considered among some of the most pristine on the planet, and in 2010 it was reported that 50% of all the healthy reefs located in the Indian ocean were found here (Everaarts, J.M. et al. 1999; Koldewey et al. 2010), and the Great Chagos Bank forms the world’s largest contiguous undamaged reef area at 12,642 km2 (Esteban et al. 2018). Indicative of their pristine status, wilderness areas such as the Chagos archipelago (Graham and McClanahan 2013) contain fish biomass levels orders of magnitude greater than anywhere else in the Indian Ocean (Head et al. 2019). Remote regions such as the Chagos archipelago likely represent coral reef ecosystems in some of their least anthropogenically influenced states. These regions likely serve and as our last true natural baselines (Esteban et al. 2018; Gorospe et al. 2018), especially as many marine ecosystems across the globe experience unprecedented rates of climate and anthropogenic change (Apprill 2020).

Coral bleaching, the process where the symbiotic relationship between the coral host and their photosynthetic dinoflagellate algae breaks-down (Brown 1997) is becoming a more frequent, and increasingly severe event (Couch et al. 2017; Sully et al. 2019), with previously unprecedented back to back bleaching events becoming common across the globe (Eakin et al. 2018; Harrison et al. 2019). Bleaching events can be the consequence of numerous stressors (e.g., Thermal stress, changes in salinity, high solar irradiance, chemical pollution, disease etc) (Rosenberg et al. 2009), because corals derive a significant portion of their nutritional requirements from these algal symbionts, prolonged bleaching can cause host mortality (Bourne et al. 2008).

Corals are complex meta-organisms, numerous layers of tissue form individual polyps, and numerous polyps are ultimately assembled into large colonies. Each of these levels hosts diverse communities of bacteria, [archaea](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/archaeon), eukaryotic [microbes](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/microorganism), and viruses (Putnam et al. 2017). These microbes play critical roles within their hosts, where they mediate nutrient cycling, metabolism and aid in pathogen defence (Lesser et al. 2004; Ritchie and Smith 2004; Peixoto et al. 2017). Traditionally, most coral biologists have ignored the role that microbes could play as the causative agents of coral bleaching, tending to focus on thermal stress and elevated irradiance, however, microbiologists developed the microbial hypothesis of coral bleaching, this posits that stresses act upon the coral host and its associated microorganisms (Kushmaro et al. 1996; Rosenberg et al. 2009). These stresses induce changes in bacterial community, which can contribute either directly, or indirectly to host bleaching (Rosenberg 2004; Rosenberg et al. 2009; Hartman et al. 2020; Meenatchi et al. 2020). It is likely that shifts in bacterial community work synergistically with thermal stresses acting directly on host algal symbionts to induce bleaching (Rosenberg et al. 2009)

Recent work is investigating the possibility that microbiome manipulation could increase coral resistance to bleaching (Rosado et al. 2019), this approach relies on the addition of microorganisms that have been putatively identified as beneficial, but determining which microorganisms are beneficial can be challenging, especially when hosts come from regions that likely already degraded and suffering anthropengic impacts. The remote Chagos archipelago provides a unique opportunity to study the bacterial communities associated with the widespread coral *Pocillopora acuta* *in-situ*, in a region of the planet that suffers negligible direct human impacts associated with increasingly urbanized coastal populations and the stresses they bring (e.g., overfishing, increased nutrient, sediment and chemical stresses). Performing work like this, in remote ecosystems that are in a state that is as close to pristine as possible allows us to determine our best estimates of how natural coral associated bacterial communities are structured. Furthermore, by examining the differential abundances of microbes from corals that span a variety of health states, from fully bleached, to what appear healthy, we can potentially glean useful insights into what could actually be beneficial coral microbes.

**Methods**

All samples were collected between 11 March 2015 and 05 May 2015, see fig 1 for details of collection locations. Small branches of *Pocillopora acuta,* generally less than 3 cm in length were collected, and as per Wainwright et al. (2019)individual branches were placed in separate sealed containers. Samples were stored at -80oC until processing occurred.

The degree to which coral colonies were bleached, unbleached or healthy was determined based upon colony colour, colour was assessed via comparisons against a colour reference card (Siebeck et al. 2006), and colonies were assigned to one of four categories (healthy, pale, very pale and bleached). Collection depth and the temperature specific to each collected colony was recorded (SI Metadata table).

DNA extraction was performed following a modified TRIzol (Invitorogen) protocol, see Mayfield et al. (2009) for full details. Briefly, 100mg of collected coral branch was placed in 500 µl of TRIzol and ground via mechanical homogenization. After RNA extraction was performed, DNA was precipitated from the aqueous phase via salting-out. DNA was re-suspended in 50 µl of TE buffer.

Pocilloporid corals display extreme levels of phenotypic plasticity, to such a degree that identifications based on morphological ID alone is difficult and unreliable, because of this we confirmed the identification of all collected samples with a gel-based restriction fragment length polymorphism (RFLP) assay (Johnston et al. 2018). Briefly, the mitochondrial open reading frame (mtORF) marker was amplified via PCR, and the resulting products where digested with the *Tsp*45I restriction enzyme to produce species specific fragment sizes that allow accurate and reliable species differentiation.

PCR amplification of microbial communities targeting the V4 region of the 16S SSU rRNA was performed using the bacterial and archaeal primers 515F and 806R (515F—GTG CCA GCM GCC GCG GTA A; 806R—GGA CTA CHV GGG TWT CTA AT), with forward and reverse primers modified to include Illumina adaptors, a linker and a unique barcode (Caporaso et al. 2011). Each reaction was performed in a total volume of 25 µl, containing 1 µl of undiluted template, 0.1 µl of KAPA 3G Enzyme (Kapa Biosystems, Inc, Wilmington, MA, USA), 0.75 µl of each primer at 10 µM, 1.5 µl of 1.5 mg ml-1 BSA, 12.5 µl KAPA PCR Buffer and water to 25 µl. PCR cycling protocol was 94 oC for 180 s, followed by 35 cycles of 94 oC for 45 s, 75 oC for 10 s, 50 oC for 60 s and 72 oC for 90 s, with a final extension at 72 oC for 10 min. Negative extraction and PCR controls were included to identify possible contamination issues.

PCR products were visualised on a 1% TBE buffer agarose gel. Normalisation and cleaning of PCR products were performed in SequalPrepTM normalisation plates (Invitrogen, Frederick, Maryland, USA) and submitted for sequencing on the Illumina MiSeq platform (600 cycles,V3 chemistry, 300-bp paired end reads) with a 30% PhiX spike (Macrogen, Korea).

Our bioinformatics workflow involved adaptor removal, quality filtering and trimming, error correction, inference of amplicon sequence variants (ASVs), removal of PhiX and chimeras, and taxonomic assignment. Details for each step are given below, and the full code for replicating the analysis can be found at <https://github.com/gzahn/Chagos>

Demultiplexed sequences were obtained from Macrogen, Korea. Barcodes and adaptors were removed with Cutadapt (Martin 2011), and reads were filtered based on quality scores and trimmed using the DADA2 package version 1.9.0 (Callahan et al. 2016) in R version 3.4.1 (R Core Team 2013). Forward reads were truncated at 240 base pairs, and reverse reads were truncated at 160 base pairs. Both forward and reverse reads were filtered to remove any reads with a max EE (expected error) of 2, and reads were additionally truncated at the end of ‘a good quality sequence’ with the parameter truncQ = 2 (see https://benjjneb.github.io/dada2/ for a detailed explanation of filtering parameters).

The DADA2 algorithm was next used to estimate error rates from all quality-filtered reads and then to merge forward and reverse reads and infer ASVs. Chimeras were removed via de novo detection. Sequenced extraction negatives were used to identify possible contaminants using the decontam R package (Davis et al. 2017), and remaining ASVs were assigned taxonomy with the RDP classifier (Cole et al. 2007) against a training set based on the Silva v132 16S database (Quast et al. 2013). Phylogenetic placement of ASVs was assigned by aligning sequence variants without an anchor using the AlignSeqs function of the DECIPHER R package version 2.6.0 (Wright 2016) and constructing a maximum likelihood tree with the optim.pml() function from an initial starting tree built using the NJ() function in the phangorn R package version 2.4.0 (Schliep 2011).

Any ASVs assigned to mitochondrial or chloroplast genomes, and those not present in at least 5% of samples were removed. Raw sequence counts were then converted to relative abundance. The Shannon diversity for each site was calculated, and non-metric multi-dimensional scaling NMDS was performed on the Bray–Curtis dissimilarity matrix of samples using the phyloseq R package version 1.25.2 (McMurdie and Holmes 2013). Permutational multivariate ANOVA (SI Table 2) was performed using the adonis() function of the vegan R package version 2.5–2 (Oksanen et al. 2013). CORNCOB package details and any specific settings, if not default

All sequences associated with this work have been deposited at the National Center for Biotechnology Information under BioProject ID: [PRJNA608014](https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA608014)

**Results**

We putatively identified 119 coral branches as *Pocillopora acuta* by visual methods collected from eight sample sites in the Chagos Archipelago (Fig-??), and identifications were confirmed by restriction fragment length polymorphism assays. We collected 94 *Pocillopora acuta* corals, and 25 *Pocillopora damicornis* coral samples*.* Unless otherwise stated all analyses were performed independently on each species. After quality filtering and merging of read, a total of 4,840,285 reads were retained for analysis, and once chimeras were removed these sequences represented 3,028 unique ASVs.

Network plots indicate, no obvious structuring of bacterial communities by island, depth or coral colony colour (SI-????). However, bleached corals do appear to harbour bacterial communities that are more similar to each other than other corals of different colony colours (SI-???), and this observation is corroborated by Permutational multivariate ANOVA showing that colony colour is significant factor influencing bacterial community structure (*p* <0.01).

Proteobacteria is the most dominant phylum in both coral species, across all islands, colony colours, and measured temperatures (SI-???). Firmicutes appear more abundant in *P. damicornis,* and boxplots demonstrating the top 10 most abundant families in each colony colour are reasonably consistent across all colours, but the mean abundance of Burkholderiaceae is higher in bleached coral colonies (SI-???)

Count Regression for Correlated Observations with the Beta-binomial (corncob) analysis indicate

----------------------------------------------------------------

No difference in bleached vs non bleached

<https://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-017-0329-8>

Not big differences in regions – nmds

<https://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-019-0776-5>

also many unidentified at genus level

Sg corals different as close to urban core – chagos remote so no terrestrial inputs

Disease also aiding bleaching Yosi Loya papers

Disbyosis - <https://mbio.asm.org/content/11/2/e02691-19>

Giant clam micfroniome – dead all have the same

<https://www.researchsquare.com/article/rs-24592/v2>

Eakin, C.M., Liu, G., Gomez, A.M., De la Couri, J.L., Heron, S.F., Skirving, W.J., Geiger, E.F., Marsh, B.L., Tirak, K.V., Strong, A.E. (2018). Unprecedented three years of global coral bleaching 2014–17. Sidebar 3.1. [in *State of the Climate in 2017*]. Bulletin of the American Meteorological Society, 99(8), S74–S75.

Apprill A (2020) The Role of Symbioses in the Adaptation and Stress Responses of Marine Organisms. Annu Rev Mar Sci 12:291–314

Brown BE (1997) Coral bleaching: causes and consequences. Coral Reefs 16:S129–S138

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. PNAS 108:4516–4522

Couch CS, Burns JHR, Liu G, Steward K, Gutlay TN, Kenyon J, Eakin CM, Kosaki RK (2017) Mass coral bleaching due to unprecedented marine heatwave in Papahānaumokuākea Marine National Monument (Northwestern Hawaiian Islands). PLOS ONE 12:e0185121

De Santo EM, Jones PJS, Miller AMM (2011) Fortress conservation at sea: A commentary on the Chagos marine protected area. Marine Policy 35:258–260

Esteban N, Unsworth RKF, Gourlay JBQ, Hays GC (2018) The discovery of deep-water seagrass meadows in a pristine Indian Ocean wilderness revealed by tracking green turtles. Marine Pollution Bulletin 134:99–105

Everaarts, J.M., Booij, K., Fischer, C.V., Maas, Y.E.M., Nieuwenhuize, J., Sheppard, C.R.C., Seaward, M.R.D., Ondersteunende Diensten (1999) Assessment of the environmental health of the Chagos Archipelago. Linnean Society Accasional Publications. Westbury Publishing,

Ferretti F, Curnick D, Liu K, Romanov EV, Block BA (2018) Shark baselines and the conservation role of remote coral reef ecosystems. Science Advances 4:eaaq0333

Gifford R, Dunne RP (2014) A Dispossessed People: the Depopulation of the Chagos Archipelago 1965–1973. Population, Space and Place 20:37–49

Gorospe KD, Donahue MJ, Heenan A, Gove JM, Williams ID, Brainard RE (2018) Local Biomass Baselines and the Recovery Potential for Hawaiian Coral Reef Fish Communities. Front Mar Sci 5:

Graham NAJ, McClanahan TR (2013) The Last Call for Marine Wilderness? BioScience 63:397–402

Graham NAJ, Wilson SK, Carr P, Hoey AS, Jennings S, MacNeil MA (2018) Seabirds enhance coral reef productivity and functioning in the absence of invasive rats. Nature 559:250–253

Harrison HB, Álvarez-Noriega M, Baird AH, Heron SF, MacDonald C, Hughes TP (2019) Back-to-back coral bleaching events on isolated atolls in the Coral Sea. Coral Reefs 38:713–719

Hartman LM, van Oppen MJH, Blackall LL (2020) The Effect of Thermal Stress on the Bacterial Microbiome of Exaiptasia diaphana. Microorganisms 8:20

Head CEI, Bayley DTI, Rowlands G, Roche RC, Tickler DM, Rogers AD, Koldewey H, Turner JR, Andradi-Brown DA (2019) Coral bleaching impacts from back-to-back 2015–2016 thermal anomalies in the remote central Indian Ocean. Coral Reefs

Head CEI, Koldewey H, Pavoine S, Pratchett MS, Rogers AD, Taylor ML, Bonsall MB (2018) Trait and phylogenetic diversity provide insights into community assembly of reef-associated shrimps (Palaemonidae) at different spatial scales across the Chagos Archipelago. Ecology and Evolution 8:4098–4107

Johnston EC, Forsman ZH, Toonen RJ (2018) A simple molecular technique for distinguishing species reveals frequent misidentification of Hawaiian corals in the genus Pocillopora. PeerJ 6:e4355

Koldewey HJ, Curnick D, Harding S, Harrison LR, Gollock M (2010) Potential benefits to fisheries and biodiversity of the Chagos Archipelago/British Indian Ocean Territory as a no-take marine reserve. Marine Pollution Bulletin 60:1906–1915

Kushmaro A, Loya Y, Fine M, Rosenberg E (1996) Bacterial infection and coral bleaching. Nature 380:396–396

Lesser MP, Mazel CH, Gorbunov MY, Falkowski PG (2004) Discovery of symbiotic nitrogen-fixing cyanobacteria in corals. Science 305:997–1000

MacNeil MA, Graham NAJ, Cinner JE, Wilson SK, Williams ID, Maina J, Newman S, Friedlander AM, Jupiter S, Polunin NVC, McClanahan TR (2015) Recovery potential of the world’s coral reef fishes. Nature 520:341–344

Mayfield AB, Hirst MB, Gates RD (2009) Gene expression normalization in a dual-compartment system: a real-time quantitative polymerase chain reaction protocol for symbiotic anthozoans. Mol Ecol Resour 9:462–470

Meenatchi R, Thinesh T, Brindangnanam P, Hassan S, Kiran GS, Selvin J (2020) Revealing the impact of global mass bleaching on coral microbiome through 16S rRNA gene-based metagenomic analysis. Microbiological Research 233:126408

Peixoto RS, Rosado PM, Leite DC de A, Rosado AS, Bourne DG (2017) Beneficial Microorganisms for Corals (BMC): Proposed Mechanisms for Coral Health and Resilience. Front Microbiol 8:

Putnam HM, Barott KL, Ainsworth TD, Gates RD (2017) The Vulnerability and Resilience of Reef-Building Corals. Current Biology 27:R528–R540

Ritchie KB, Smith GW (2004) Microbial Communities of Coral Surface Mucopolysaccharide Layers. In: Rosenberg E., Loya Y. (eds) Coral Health and Disease. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 259–264

Rosado PM, Leite DCA, Duarte GAS, Chaloub RM, Jospin G, Rocha UN da, Saraiva JP, Dini-Andreote F, Eisen JA, Bourne DG, Peixoto RS (2019) Marine probiotics: increasing coral resistance to bleaching through microbiome manipulation. ISME J 13:921–936

Rosenberg E (2004) The Bacterial Disease Hypothesis of Coral Bleaching. In: Rosenberg E., Loya Y. (eds) Coral Health and Disease. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 445–461

Rosenberg E, Kushmaro A, Kramarsky-Winter E, Banin E, Yossi L (2009) The role of microorganisms in coral bleaching. ISME J 3:139–146

Sheppard C, Ateweberhan M, Chen A, Harris A, Jones R, Keshavmurthy S, Lundin C, Obura D, Purkis S, Raines P, Riegl B, Schleyer M, Sheppard A, Tamelander J, Turner J, Visram S, Yang S-Y (2013) Coral Reefs of the Chagos Archipelago, Indian Ocean. pp 241–252

SHEPPARD CRC, ATEWEBERHAN M, BOWEN BW, CARR P, CHEN CA, CLUBBE C, CRAIG MT, EBINGHAUS R, EBLE J, FITZSIMMONS N, GAITHER MR, GAN C-H, GOLLOCK M, GUZMAN N, GRAHAM NAJ, HARRIS A, JONES R, KESHAVMURTHY S, KOLDEWEY H, LUNDIN CG, MORTIMER JA, OBURA D, PFEIFFER M, PRICE ARG, PURKIS S, RAINES P, READMAN JW, RIEGL B, ROGERS A, SCHLEYER M, SEAWARD MRD, SHEPPARD ALS, TAMELANDER J, TURNER JR, VISRAM S, VOGLER C, VOGT S, WOLSCHKE H, YANG JM-C, YANG S-Y, YESSON C (2012) Reefs and islands of the Chagos Archipelago, Indian Ocean: why it is the world’s largest no-take marine protected area. Aquatic conservation : marine and freshwater ecosystems 22:232–261

Siebeck UE, Marshall NJ, Klüter A, Hoegh-Guldberg O (2006) Monitoring coral bleaching using a colour reference card. Coral Reefs 25:453–460

Sully S, Burkepile DE, Donovan MK, Hodgson G, Woesik R van (2019) A global analysis of coral bleaching over the past two decades. Nat Commun 10:1–5

Wainwright BJ, Afiq-Rosli L, Zahn GL, Huang D (2019) Characterisation of coral-associated bacterial communities in an urbanised marine environment shows strong divergence over small geographic scales. Coral Reefs