Running title: ABCDom I

**Thermal stress and bleaching alter coral DOM exudation and enrich distinct, copiotrophic microbial communities**

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**Abstract**

**Whatever is typed here in bold is super important because it will be the abstract that we still need to write in total. But having some bold words on paper just looks very interesting. What is also interesting are our findings in this great piece of work. It is pretty awesome to see that healthy corals at when heated and reach their accumulated stress threshold and start to bleach, that they dump a bunch of DOM, which is also different from healthy coral DOM. Next to that it promotes the growth of nasty bacterial lineages. I think this abstract can be even a bit longer so I am just going to type some extra lines to make it look pretty. I hope you enjoy reading this written word vomit. However what we might be able to agree on is the things that we should put in the abstract. The opening is important because we want to grab the attention of the reader so let’s not do a sentence that you read all the time, for instance that coral bleaching will be occurring more and more often. Or at least not in this way. Maybe we can start more dramatically: With humanity failing to dramatically reduce their CO2 output, and thereby enhancing the world’s greenhouse effect, and rapidly increasing earth's sea surface water temperatures, it is inevitable that coral bleaching will happen more often and with shorter intervals. Therefore there is a need to understand the effect of coral bleaching on the smallest scales: on molecular and microbial levels. The last decades molecular techniques have granted us insight into the effects of dissolved molecules released by benthic primary producers such as corals and algae. However, recent techniques allow for gaining insight in the cacophony of molecules from which these microbes get their energy. Here we use 16S amplicon sequencing combined with untargeted metabolomics to assess the effects of bleaching and temperature stress on the release of DOM and the microbial community response. During the bleaching event of 2019 in Mo'orea, French Polynesia bleached and until then, resistant and healthy looking corals, were collected and subjected to 7 days of ambient or elevated temperatures. Subsequently they were used to harvest produced and eluded dissolved organic matter during a 3 hour incubation. The released DOM was used as a medium for microbial dilution cultures. Heated corals that started to bleach released more dissolved carbon than the healthy corals kept at ambient temperatures resulting in higher microbial growth rates and concentrations. Untargeted metabolomics revealed that the molecular makeup of the released dissolved organic matter is different which explains the promotion of bacterial lineages associated with the microbilization of coral reefs. SO, that are quite some words I typed. And it will probably not even end up in our abstract. So this is def. a placeholder.**

The coral reef benthos harbors a patchwork of organisms, especially scleractinian coral and algae, which exert measurable influence on the surrounding water column biogeochemistry. On healthy coral reefs, scleractinian corals influence water column dynamics via multiple mechanisms including heterotrophic feeding and importantly, dissolved organic matter (DOM) exudation. Benthic primary producers on coral reefs, namely corals and algae, have been shown to release upwards of 50% of their daily photosynthate in the water column in the form of DOM, which can in turn serve as an energy source and/or chemical cue for water column bacteria [(Ducklow, 1990; Haas et al., 2011)](https://www.zotero.org/google-docs/?ZezKAM). DOM exudates from corals are chemically distinct from the surrounding seawater and DOM is released from corals at higher rates and elevated concentrations compared to the surrounding sweater [(Haas et al., 2013; Nelson et al., 2013)](https://www.zotero.org/google-docs/?CmmHgv). Qualitatively, DOM exudates from corals contain a unique cocktail of chemicals, with distinct fDOM (fluorescent DOM), DCNS (dissolved combined natural sugars) and exometabolome profiles compared to seawater [(Kelly et al., 2022; Nelson et al., 2013; Quinlan et al., 2018)](https://www.zotero.org/google-docs/?LQLag3).

Upon exudation, coral DOM facilitates the interaction between the coral holobiont and the surrounding bacterioplankton. Coral reef bacterioplankton exhibit chemotactic responses to a variety of DOM, including dimethylsulfoniopropionate (DMSP) [(Tout et al., 2015)](https://www.zotero.org/google-docs/?8XC7iQ). Known coral bacterial pathogens have also been shown to chemotax towards coral exudates, further indicating the crucial role that this zone of interaction may play in mediating coral health [(Garren et al., 2014)](https://www.zotero.org/google-docs/?FrnmQ4). Coral DOM exudates also support the growth and activity of distinct heterotrophic bacterioplankton communities [(Haas et al., 2011, 2013; Nelson et al., 2013; Silveira, Cavalcanti, et al., 2017)](https://www.zotero.org/google-docs/?HxBjKp). Both laboratory and *in situ* studies have identified unique metabolite and microbial communities adjacent to corals compared to the surrounding seawater [CITE]. The microbial communities are often enriched in genes related to dynamic processes including chemotaxis, motility, and signal transduction, suggesting that these “spheres” surrounding corals contain bacterioplankton with the capacity to interact with the coral reef benthos [(Ochsenkühn et al., 2018; Silveira, Gregoracci, et al., 2017; Tout et al., 2014; Walsh et al., 2017; Weber et al., n.d.)](https://www.zotero.org/google-docs/?gwUPnC). Clearly, this zone of interaction between corals and bacterioplankton is an essential area for benthic-pelagic coupling and necessary to consider when feedback loops between the benthos and the water column in the coral reef ecosystem. However, despite our burgeoning knowledge of how healthy corals influence reef water column dynamics via DOM exudation, we know relatively little about if and how this relationship changes when corals are stressed.

The ocean, which is estimated to have absorbed 90% of the earth’s extra heat since 1955, is warming rapidly [(Levitus et al., 2012; *Technical Summary — Special Report on the Ocean and Cryosphere in a Changing Climate*, n.d.)](https://www.zotero.org/google-docs/?f20Bee)(Levitus et al., 2012; IPCC, 2019: Technical Summary, IPCC 2022 ; Chapter 3). Ocean warming is a global stressor which can damage reefs worldwide. Thermal stress harms corals via bleaching, a well-documented and widespread phenomenon in which the symbiosis between corals and Symbiodinacaeae breaks down if corals are exposed to elevated temperatures for a certain duration of time [(Brown, 1997)](https://www.zotero.org/google-docs/?uwbEmc). Although corals can recover from bleaching, if thermal stress persists for an extended period, the coral will die [(Hoegh-Guldberg, 1999)](https://www.zotero.org/google-docs/?WSpdAe). Mass coral bleaching events have been recorded in the past but appear to occur more frequently, giving coral reefs less time to recover [(Heron et al., 2016; van Hooidonk et al., 2016](https://www.zotero.org/google-docs/?b9wRMu), [UN report][)](https://www.zotero.org/google-docs/?b9wRMu). Coral bleaching, even at sub-lethal levels, alters the coral holobiont’s metabolism and significantly shifts the community structure of its bacterial partners [(McDevitt-Irwin et al., 2017)](https://www.zotero.org/google-docs/?idh2aw). However, there has been very little research on the implications of coral bleaching for reef water column dynamics.

Preliminary evidence suggests that coral-water column interactions are altered during periods of “stress”, including elevated temperatures. Bacteria are able to sense chemical cues released by corals experiencing stress. In one study, the bacterial pathogen *Vibrio coralliilyticus* used dimethylsulfoniopropionate (DMSP) released at elevated concentrations by heat stressed corals as a chemotactic cue to target these organisms [(Garren et al., 2014)](https://www.zotero.org/google-docs/?GGAogg). Additionally, Niggl et. al 2009 found that bleaching corals released elevated levels of particulate nitrogen (PN) and particulate organic carbon (POC) into the water column [(Niggl et al., 2009)](https://www.zotero.org/google-docs/?snQDmj). Given that healthy corals exude DOM into the surrounding seawater and influence subsequent bacterioplankton dynamics [CITE], it is reasonable to posit that elevated temperatures, which can dramatically alter coral physiology, will result in altered DOM exudation and potentially altered water column microbial dynamics.

To address these crucial knowledge gaps, we made use of historically bleached and healthy corals from Mo'orea, French Polynesia. In 2019 the reefs of Mo'orea experienced a mass bleaching event after a prolonged period of massive heat anomalies [(Leinbach et al., 2021; Speare et al., 2021)](https://www.zotero.org/google-docs/?FfnWKS). The reefs experienced frequently sea surface temperatures higher than the thermal stress accumulation threshold of 29°C, from December 2018 till May 2019 [(Leinbach et al., 2021; Speare et al., 2021)](https://www.zotero.org/google-docs/?vuiESC). At the start of April 2019 seawater temperatures reached the max of 30°C and that month the first bleaching was observed. After temperatures lowered in May, some corals showed signs of recovery in August, and were reported to be recovered in October using bleaching scores [(Leinbach et al., 2021)](https://www.zotero.org/google-docs/?rfzi5O).

Corals collected in May 2019 were placed in a flow-through aquaria system to test the combined and independent effects of thermal stress and bleaching on coral DOM exudation and subsequent bacterial remineralization and growth. We use a combination of traditional oceanographic techniques, high throughput amplicon sequencing, and untargeted metabolomics to quantitatively and qualitatively assess differences in DOM exudates from corals in different bleaching states, and if these different DOM pools yielded different bacterioplankton communities. These data present a comprehensive assessment of how elevated temperatures induce altered DOM exudation in corals, and in turn how it influences reef bacterioplankton community structure. Showing how actively bleaching corals do not only release more carbon, but the molecular makeup is significantly different, leading to enrichment of distinct, copiotrophic microbial communities. Therefore mass bleaching events have the potential to induce the shift of ecosystem trophic structure towards higher microbial biomass and energy use; the microbialization of coral reefs.

**METHODS**

**Mo'orea 2019 Bleaching Event**

The reefs of Mo'orea, French Polynesia suffered from a massive bleaching event in April 2019, with thermal anomalies since December 2018 [(Leinbach et al., 2021)](https://www.zotero.org/google-docs/?U2Lhlx)]. Mo'orea Coral Reef Long Term Ecological Research (MCR LTER) daily average water temperature data (Fig 1B) time series was combined from 3 sites on the MCR LTER fore reef: FOR1, FOR4 and FOR5 (GPS location: 17°28'30.0"S 149°50'13.2"W; 17°32'49.2"S 149°46'08.4"W; 17°34'55.2"S 149°52'30.0"W; respectively). From each location data from five sensors (“upper water column”, “middle water column”, “bottom water column”, “temperature shallow”, and “temperature deeper”) was used to calculate the average temperature +/- 1 standard deviation. The coral bleaching threshold for these sites was determined to be 29 °C [(Leinbach et al., 2021; Pratchett et al., 2013)](https://www.zotero.org/google-docs/?udarhF).

**Experimental Design**

*Field Collection (Fig 1A)*

Coral nubbins from three different species (*Pocillopora verrucosa*, *Acropora pulchra*, and *Porites rus)* were collected in Mo'orea, French Polynesia immediately following a large-scale thermal anomaly, with temperatures exceeding the coral bleaching threshold by upwards of 1°C (Figure 1B). *Pocillopora verrucosa* and *Acropora pulchra* were collected from a common garden LTER1 site on the forereef of Paopao Bay, Mo'orea, French Polynesia (17°30'36.7"S 149°51'10.3"W). *Porites rus* nubbinswere collected from LTER 2 back-reef (17°28'12.0"S 149°47'45.6"W). Coral nubbins were collected under permits issued by the French Polynesian Government (Délégation à la Recherche) and the Haut-commissariat de la République en Polynésie Francaise (DTRT) (Protocole d'Accueil 2005-2021). Coral nubbins from the three species were visually inspected at the time of collection and assigned as either “Non-bleached” or “Bleached” phenotypes. After collection, corals were transported to the GUMP research facility and acclimated to ambient conditions in a 1300L flow-through water table for 3 days prior to the experiment. Coral bleaching status was again validated after this 3-day acclimation period with a further visual inspection and assessment of Symbiodiniaceae cell densities via flow cytometry (Fig 1C).

*Pre-Treatment in Flow-Through Aquaria*

At the start of the pre-treatments, coral nubbins were secured to silicon holders and placed into acid-washed (10% HCl and triple milliQ water rinse) 1.5L polycarbonate aquaria containing 1.5L unfiltered water. To mimic reef-wide bleaching/thermal stress signals, the 3 species of corals were combined in each aquaria: 2 nubbins from the 3 coral species in a given bleaching phenotype were combined in individual aquaria. Influent water from the GUMP Station flow-through seawater system was pumped into the aquaria at a constant rate using a masterflex L/S digital display peristaltic pump connected with 1/8th” platinum cured silicone tubing and master flex two-stop tubing, yielding a final residence time of 5 hours in the aquaria. Water in each aquaria was recirculated with 4.8W pumps moving 240 L hr-1. Aquaria containing Non-bleached and Bleached nubbins were exposed to 7 days of either ambient (28.6°C) or elevated water temperatures (32˚C +/- 0.2˚C) and ambient light levels, yielding 4 treatments representing a factorial cross of bleaching phenotype and temperature: “Non-bleached + Ambient”, “Non-bleached + Heated”, “Bleached + Ambient”, and “Bleached + Heated”. Additionally, 2 water-only control treatments for both temperatures were included in the pre-treatments (“Ambient Water Control” and “Heated Water Control”). All aquaria pre-treatments were performed in triplicate. Aquaria were heated using four 300 W and two 800 W Finnex heaters.

*DOM Exudation*

On the day of the experiment, the flow through of unfiltered water and the recirculation of water within the aquaria was stopped. Water was removed from each aquaria until 400 mL remained. 800 mL .22µm filtered offshore water at ambient temperature was then added to yield a final volume of 1200 mL for the DOM exudations. Corals were left in the 1.5L aquaria to exude DOM for 3 hours (15:00 h - 18:00 h) while heat treatments were maintained. After 3 hours, DOM exudates were collected by filtering the 1200 mL of aquaria water through a .22µm sterivex filter and into 2 L polycarbonate bottles (10% hydrochloric acid washed). To prevent DOM contamination from the sterivex unit, each sterivex was flushed with filtered offshore water. Following exudation, corals were removed from the aquaria and airbrushed to collect tissue slurry for downstream DNA sequencing and Symbiodiniaceae quantification.

*Dilution Cultures (Fig 1A)*

Filtered DOM exudates were used as growth media for dark incubation dilution cultures, while unfiltered backreef water was used as an inoculum representative of ambient backreef bacterioplankton communities [(Nelson et al., 2013)](https://www.zotero.org/google-docs/?tWnZ0T). 1200 mL of DOM media from each replicate aquaria (n=3 per treatment) was mixed with 400 mL bacterioplankton inoculum (3:1 volumetric ratio) via inversion in a 10% hydrochloric acid washed 2 L polycarbonate bottles. Dilution cultures were then split equally into two, 1 L 10% hydrochloric acid washed polycarbonate bottles (800 mL culture per bottle). This yielded a total of n=2 technical replicates per biological replicates, and a total of n=3 biological replicates per DOM treatment (“Non-bleached + Ambient”, “Non-bleached + Heated”, “Bleached + Ambient”, and “Bleached + Heated” and the two water control treatments “Ambient Water Control” and “Heated Water Control”). One of the “Non-bleached + Heated” bottles was lost yielding a n=2 for this treatment. Half of the technical replicates were immediately sacrificed at the beginning of culturing (T0), while the remaining technical replicates were incubated in the dark at ambient temperatures for 36 hours.

**Sample Collection**

*Symbiodiniaceae quantification:* To assess bleaching status of the corals during collection and at the end of the 7 day incubation and exudation experiment, coral nubbins were sacrificed. Coral nubbins were frozen and airbrushed. Slurry collected with airbrushing was analyzed using flow cytometry.

*Microbial abundance:* Samples for flow cytometry were taken at the start of the incubation and at 2, 8, 16, 20, 24, 32, and 36 hours. At every time point, 1 mL of each sample was fixed with 16 μL of 32% paraformaldehyde and stored at -40 °C for 14 days prior to transportation to University of Hawaiʻi at Mānoa campus where they were frozen at -80 °C for 6 months prior to flow cytometry processing.

At T0 and Tend water (800 mL) was sampled for microbial communities, dissolved organic carbon (DOC), and solid phase extraction of dissolved organic matter using a peristaltic pump (Masterflex L/S, Cole-Parmer) connected to 10% hydrochloric acid cleaned and seawater leached silicon tubing.

*Microbial community:*

To assess bacterioplankton community shifts, 800 mL of sample water was passed through a .22µm sterivex to collect bacterioplankton for downstream DNA analysis. Sterivex filters were frozen at -40 °C for 14 days prior to transportation to University of Hawaiʻi at Mānoa campus where they were frozen at -80 °C for 6 months prior to DNA extraction.  
  
*Dissolved organic carbon:*

35 mL of .22µm sterivex filtrate was collected in 10% hydrochloric acid washed, combusted, triple sample-rinsed amber glass vials for DOC analysis. Care was made to flush each sterivex with about 50 mL of sample water prior to collecting DOC to avoid contamination. DOC samples were then acidified with 50µL of 4N hydrochloric acid to yield a pH of less than 2 and kept at room temperature for 14 days prior to transportation to the University of Santa Barbara (USA). DOC samples were stored at 15 °C in the dark for 6 months prior to sample processing.

*Solid phase extraction of dissolved organic matter*

The remaining .22µm sterivex filtrate was collected in 10% hydrochloric acid washed, triple sample rinsed 1 L polycarbonate bottles and acidified with HCl to pH < 2.

Remaining volumes were equalized to 700 mL per bottle, two bottles who had less volume were equalized to 550 mL. After equalizing, 50 mL of the acidified sample water was used to flush the lines prior to the solid phase extraction. Resulting in 650 mL or 500 mL of sample water for solid phase extraction. Dark incubation backreef inoculum (700 mL) was sampled in tandem with the treatment sampling at T0.

**Sample Processing**

*Flow cytometry*

*Microbial abundance:* 1mL microbial abundance samples were thawed, 200µL of each sample was stained with 2µL 100X SYBR Green, and samples were run on a Attune Flow Cytometer to enumerate bacterial cell counts.  
 *Symbiodiniaceae abundance:* who knows. magic?

*DOC Sample processing:*

All samples were processed and analyzed via high-temperature combustion on slightly modified Shimadzu TOC-V analyzers at UCSB according to the protocol outlined in Carlson et al., 2010.

*Microbial community DNA extraction, library prep, and sequencing*

Sample DNA extraction protocols followed those outlined in Bullington et al., 2022 [(Bullington et al., 2022)](https://www.zotero.org/google-docs/?OXU4cy). To extract bacterial DNA from the sterivex filters, the filter was removed from the plastic casing using sterile pliers, scalpels, and tweezers, and added to MP Biomedicals Lysing Matrix A (No. 116910100) tubes with 0.5 mL MC 1 lysis buffer and homogenized using a MP Biomedicals FastPrep-96 bead beater. A portion of the homogenate (0.4 mL) was recovered and DNA extractions were completed using the Macherey-Nagel NucleoMag Plant Extraction Kit (No. 744400.4) with KingFisher Accessory Kit (No. 744951). Samples were eluted to a final volume of 110 μL.

Amplicon sequencing of the V4 16S rRNA gene region was conducted on an Illumina MiSeq at the University of Hawaiʻi at Mānoa Advanced Studies in Genomics, Proteomics and Bioinformatics facility. A single barcode library preparation approach with Golay barcoded forward primers and non-barcoded reverse primers was used to target the V4 region of the 16S rRNA gene. 515F and 806R Earth Microbiome Project primers were used [(Apprill et al., 2015; Caporaso et al., 2011; Parada et al., 2016)](https://www.zotero.org/google-docs/?gJnOox). Amplicons were generated from a single round of PCR using primers that include Illumina spacers, Illumina adapters, index sequences (on the forward primers), and 16S rRNA gene template region. PCR reagents included 7.2 ul DNase free water, 10 ul PLAT II MM (2X) (Invitrogen, Platinum II Hot-Start PCR Master Mix, Catalog No: 14000012), .4 ul Forward primer 10 uM, .4 ul Reverse Primer 10 uM, and 2 ul DNA template. PCR was performed on an Applied Biosystems SimpliAmp (Catalog No: A24811) system using an initial denature of 94⁰C for 2:00 min followed by 35 cycles of 94°C for 15 seconds, 54°C for 15 seconds, 68°C for 7 seconds, followed by a final extension at 68°C for 3 minutes. DNA extraction blanks and no-template control blanks were included as negative controls and mock communities (ZymoBIOMICS Microbial Community DNA Standard, Cat No: D6305) were included as positive controls to enable discernment of contaminants from kits or processing. Method blanks had substantially lower sequence read depth (mean = 1,590 reads/sample) than samples (mean = 88,681), with samples ranging from 12,609 reads/sample to 155,685 reads/sample. Total amplicons per sample were normalized to between 1.25 and 1.5 ng/uL using Charm Biotech Just-a-Plate PCR purification and normalization kit. Amplicons were pooled and sequenced using an Illumina MiSeq V3 600 paired-end cycle run at the University of Hawaiʻi at Mānoa Advanced Studies in Genomics, Proteomics and Bioinformatics facility. All samples were amplified and sequenced in duplicate technical replicates.

*Dissolved Organic Matter*

PPL cartridges were eluted with MeOH. Extracts were dried down with a vacuum centrifuge (brand XX) and redissolved with 70 uL 80% MeOH 1% FA. The two samples who had less volume were redissolved to 50 uL. 5uL of sample was injected bla bla bla. And the settings of the orbitrap were xxx

**Data Processing and Analysis:**

*16S Amplicon Bioinformatics*

16S sequences were processed using the bioinformatic pipeline outlined in Arisdakessian et. al 2020 [(Arisdakessian et al., 2020; Jani et al., 2021)](https://www.zotero.org/google-docs/?rfUhc6). Raw paired fastq reads were preprocessed using the dada2 R package. We truncated reads at position 220 (190 for the reverse read) and discarded them if they contained a number of expected errors above 3 using the filterAndTrim() function. Denoising was performed with the learnError() and dada() functions with default parameters. Using the mergePairs() function, we merged reads if they overlapped by at least 20 bases, and allowed for 1 mismatch at most. Triplicate technical replicates were then merged bioinformatically. We used mothur [(Schloss et al., 2009)](https://www.zotero.org/google-docs/?UeJQlI) along with the Silva (release 132) database [(Quast et al., 2013)](https://www.zotero.org/google-docs/?8IJ4OC) to align and annotate the sequences. Sequences with a start or stop position outside the 5th-95th percentile range (over all sequences) were discarded . We removed potential chimeras with chimera.vsearch() . Taxonomies were assigned using classify.seqs() and classify.otus(). We removed all mitochondrial or chloroplast OTUs, as well as sequences with no annotations at the domain level. Using sub.sample(), we normalized the abundance in each sample by subsampling 12,000 sequences from each. Samples with less than this amount were discarded. OTUs were defined as unique “amplicon sequence variants” by dada2; we used the lulu R package to refine OTUs [(Frøslev et al., 2017)](https://www.zotero.org/google-docs/?TAFwa4): We merged two OTUs if all of the 3 following conditions were satisfied: 1) They co-occur in every sample, 2) One of the two OTUs has a lower abundance than the other if every sample and 3) they share a sequence similarity of at least 97% . Finally, we discarded OTUs with a total abundance of 2 or less.

*Metabolomics Bioinformatics*

The RAW files were converted to .MZml files using MSConvert [(Chambers et al., 2012)](https://www.zotero.org/google-docs/?Yn5G33). [insert settings from IRINA] and then we clean the data - blank removal and transient feature removal which results in the exometabolome samples.

*Statistics*

Data analysis and statistics were done using R (version 4.1.2). Main packages used are the core packages within tidyverse [(Wickham et al., 2019)](https://www.zotero.org/google-docs/?rpsgge) , vegan [(Oksanen, 2013)](https://www.zotero.org/google-docs/?8nQdJr) , BiodiversityR , pairwiseAdonis [cite], "stats" [cite]. Scripts and additional packages are available through https://github.com/NIOZ-DOM-Analysis/ABCDom

**RESULTS**

**Water temperatures in 2018 and 2019 (Figure 1B)**

Bleaching was first observed in the corals adjacent to Gump Station, Moorea, in April 2019 [(Leinbach et al., 2021)](https://www.zotero.org/google-docs/?2lb6Gy). Prior to this there were multiple heat anomalies starting in December 2018 in which temperatures exceeded the thermal stress accumulation threshold level of 29 °C [(Leinbach et al., 2021)](https://www.zotero.org/google-docs/?95Hbhs). By the start of field collection in May 2019, the temperatures dropped under the 29 °C threshold. Corals had experienced 110 days with temperatures exceeding the threshold in a period of 6 months (151 days).

**Symbiodiniaceae Densities (Figure 1C and 1D)**

Symbiodiniaceae cell densities were used to assess individual and aquaria wide bleaching levels of corals collected in the field and after the 7 day pre-treatment, respectively. As expected, field collected non-bleached corals had significantly higher Symbiodiniaceae cell densities than field collected bleached corals (ANOVA, F=45.552, p=2.67e-08). Additionally, coral species (ANOVA, F=4.738, p=0.0137), the interaction between species, and bleaching level (ANOVA, F=4.287, p=0.0199) were also significant. After 7 days of flow through aquaria incubation at elevated and ambient temperatures, the 4 coral treatments had varying degrees of bleaching/paling (measured as averaged Symbiodiniaceae densities in a given aquaria), although these differences were not statistically significant (one-way ANOVA, F=2.623, p=0.123) the “Non-bleached + Ambient” treatment, which reflected the aquaria system control for “Healthy”, Non-bleached nubbins, maintained the highest Symbiodiniaceae densities. “Non-bleached + Heated” aquaria exhibited slightly lower Symbiodiniaceae cell densities consistent with some paling, yet still maintained higher cell densities than their “Bleached + Ambient” and “Bleached + Heated” aquaria counterparts. The additional 7 days of accumulating thermal stress caused the previously healthy looking corals to lose part of their symbiont densities, and therefore in the process of (active) bleaching. As expected, bleached aquaria (“Bleached + Ambient” and “Bleached + Heated”) had lower Symbiodiniaceae densities. However, the “Bleached + Heated” aquaria had slightly higher average Symbiodiniaceae densities than “Bleached + Ambient”. This is likely because aquaria which had dead coral nubbins were excluded; thus the remaining “Bleached + Heated” aquaria that were included in our experiment had similar Symbiodiniaceae densities to their “Bleached + Ambient” counterparts.

**DOC (Figure 2A)**

DOC exudation appeared to be affected by treatment, we were unable to elucidate significant differences, likely due to low sample size and short exudation times. Non-bleached but thermally stressed corals (“Non-bleached + Heated”) and bleached corals (“Bleached + Ambient”) exhibited higher surface area normalized DOC values compared to the healthy coral controls (“Non-bleached + Ambient”), whereas corals that were both bleached and thermally stressed (“Bleached + Heated”) exhibited lower surface area normalized DOC values than the healthy controls. Despite these differences, treatment did not have a significant effect, likely due to reduced sample size due to DOC contamination (Kruskal-Wallis chi-squared=4.1667, p=0.244). The effect of treatment was found to be marginally significant on raw DOC values for all coral treatments and water controls (Kruskal-Wallis chi-squared=9.3187, p=.09), with coral treatments generally having higher DOC concentrations than the water controls (Figure S1).

**Metabolomes (Figure 2B)**

Untargeted metabolomics was used to assess compositional differences between the exudates from different treatments. The complete exometabolomes were used to generate a Bray-Curtis dissimilarity matrix to test and visualize multivariate differences in metabolomes between the various treatments. Metabolomes were significantly affected by treatment, indicating that different bleaching/thermal stress levels in corals produced compositionally distinct DOM exudates (PERMANOVA, F=5.8552, R2=0.70927, p≤.001, Table S1).

Due to low sample sizes for each treatment, pairwise adonis comparisons revealed no significant pairwise differences between DOM treatments (p>.05 after p-value correction for multiple comparisons, Table S2). However, R2 values (Figure S2) and visualizations (Figure 2B and S3) indicate substantial differentiation between the different treatment metabolomes. Specifically, the two heated coral treatments (“Bleached + Heated” and “Non-bleached + Heated”) were very similar to each other (24.1% different) and distinct from all other treatments, while the other coral treatments (“Bleached + Ambient” and “Non-bleached + Ambient”) and the two water controls all had moderately distinct clusters. Within the remaining treatments, “Non-bleached Ambient” coral controls and “Ambient Water Controls” were the most similar (33.3% different), followed by the two ambient temperature coral treatments (“Bleached + Ambient” and “Non-bleached + Ambient”, 35.1% different). The “Bleached + Ambient” treatment remained distinct from the “Ambient Water Control” (51% different) and the “Heated Water Control” was distinct from all other treatments.

Treatment had a significant effect on metabolomes when comparing only the coral DOM samples, further confirming that stressed corals exuded compositionally distinct DOM (F=4.9058, R2=0.65, p≤.001).

Metabolomic samples were also analyzed on their entropy, evenness and richness (figure S4). Exometabolome richness was not normally distributed (Shaprio-test, W= 0.88, p =

0.03). The water controls were significantly lower in richness (Kruskal Wallis chi-squared: 13.57, p = 0.02) except there was no significant difference between “Heated Water Control” and “Non-bleached + Ambient” treatment. The 4 treatment groups showed no significant difference in richness except for "Bleached + Heated” vs “Non-bleached + Ambient".

The entropy, or H’diversity of the exometabolomes was normally distributed (Shapiro - test, W = 0.96, p = 0.63), but showed differences between groups (ANOVA: F = 15.82, p < 0.01), most significant differences were between the heated and the ambient treatments. Between the heated treatments there was no significant difference. Within the ambient treatments there was only a significant difference between “Bleached + Ambient” and “Ambient water control (p = 0.03). Only “Bleached + Ambient” was not significantly different with the two headed treatments. Pilou’s evenness showed the same significant differences between groups as the H’ Diversity. A full overview of the statistical tests and results for the entropy, evenness and richness can be found in supplement table X

**Bacterial Growth (Figure 3)**

Flow cytometry revealed distinct bacterial growth patterns between the DOM treatments. Bacterial cell concentrations generally peaked after 24 hours of growth in dark bottle incubations and differed significantly between treatments (one-way ANOVA, F=6.82, p=0.004). Generally, coral DOM treatments yielded higher bacterial concentrations than the water controls. Within the coral DOM treatments, “Non-bleached + Heated” DOM yielded significantly higher bacterial concentrations than the control “Non-bleached + Ambient” DOM treatment (Tukey post-hoc test, p<.05), with bacteria grown on “Non-bleached + Heated” DOM reaching concentrations of 3160 cells/µL (∓ 790), nearly double that of the ““Non-bleached + Ambient” DOM treatment (1562 ∓ 137).

Bacterial specific growth rate, in natural log of bacterial cells per hour, also differed significantly between the treatments (one-way ANOVA, F=6.363, p=0.005). All 3 of the stressed coral DOM treatments yielded higher specific growth rates compared to the “Non-bleached + Ambient” coral control and water controls. The significant effect of treatment on bacterial specific growth rate was predominantly driven by the low specific growth rate in the hot water control treatment, which was significantly lower than all 4 of the coral DOM treatments (Tukey post-hoc test, p<.05).

Bacterial growth efficiency (Figure S5), measured as the percentage of total consumed carbon that was incorporated into bacterial biomass, was only marginally significantly affected by treatment (ANOVA, F=3.849, p=0.0859). While we lacked the statistical power to detect a significant effect of treatment, at least partially because of lost samples due to DOC contamination, it appears that bacteria in the “Non-bleached + Ambient” and “Bleached + Ambient” treatments had comparable BGE that was roughly 3-4% higher than the BGE of the “Non-bleached + Heated” treatment. Additionally, bacteria grown on “Heated Water Control” DOM had a much lower BGE than their “Ambient Water Control” counterparts. BGE for “Bleached + Heated” was not able to be calculated due to DOC contamination at the final timepoint.

**Microbial Community Structure (Figure 4)**

Not only did the different treatments exude different DOM, but this DOM grew distinct microbial communities. There was a clear change in microbial communities from the start to the end of the bottle incubations, indicating that distinct communities grew that were not simply reflective of the starting communities (one-way PERMANOVA, F=72.033, R2=0.71, p≤.001, Figure S6). Unifrac dissimilarity data from 16S amplicon sequences was used to assess the effect of DOM treatment on microbial community structure after 36 hours of growth. Treatment significantly affected bacterioplankton community structure (one-way PERMANOVA, F=4.637, R2=0.72, p≤.001, Table S3).

Due to low sample sizes for each treatment, pairwise adonis comparisons revealed no significant pairwise differences between DOM treatments (p>.05 after pvalue correction for multiple comparisons, Table S4). However, R2 values (Figure S7) visualizations (Figure 4 and S8) indicate substantial differentiation between a) water control DOM communities (both ambient and high) and coral DOM communities and b) differences between control “Non-bleached + Ambient” coral DOM communities and the three other coral DOM communities. Specifically, the two water control communities clustered tightly together (39.9% different) yet maintained a high degree of differentiation from the 4 coral DOM communities (mean difference of 79.4%). The “Non-bleached + Ambient” coral controls maintained a distinct, intermediate cluster between the water controls (mean difference of 62.6%) and the 3 stressed coral treatments (mean difference of 58.9%). There was a substantial degree of overlap in microbial community structure between the 3 stressed coral treatments, with a mean difference of 29.7% between the communities.

Treatment had a significant effect on community structure when comparing only the coral DOM samples, further confirming that DOM exudates from stressed corals enriched distinct bacterioplankton communities (F=2.822, R2=0.59, p=.009).

Microbial community alpha diversity metrics (observed sequences, Chao diversity, Shannon diversity, and Shannon’s Evenness) were assessed after 36 hours of growth and found to generally not be significantly affected by treatment (Figure S9). Observed sequences, Chao diversity, and Shannon’s evenness were all not significantly affected by treatment (p≥.05, K-W test for observed sequences and ANOVA for Chao diversity and Shannon’s evenness). Shannon diversity was significantly affected by treatment (ANOVA, p=0.0366, F=3.914). Although tukey post-hoc testing revealed no significant pairwise differences, the “Non-bleached + Ambient” treatment had the highest Shannon diversity, while the “Non-bleached + Heated” and “Heated Water Control” treatments had the lowest.

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**Metabolome-Microbiota Correlations (Figure S10).**

Procrustus and mantel test placeholder

**Differential Abundance ASV Analysis (Figure 5).**

Multivariate analysis clearly showed that bleached/thermally stressed corals enriched distinct bacterioplankton communities compared to their healthy “Non-bleached + Ambient” counterparts. In order to directly elucidate which specific bacterial taxa in the plankton were driving these differences, we performed DESEq2 on a subset of the data that only included the 4 coral DOM treatments. DESEq2 was run on raw read counts prior to the subsampling and Lulu steps of our bioinformatic pipeline. ASVs were culled so that only those with raw abundance ≥ 50 in three or more samples or raw abundance ≥ 1000 in one or more samples were included, which comprised a subset of 187 ASVs. Given that the abundance of ASVs was going to be compared between the 3 stressed coral treatments and the “Non-bleached + Ambient” coral controls, we further subset ASVs that exhibited consistent behavior within the “Non-bleached + Ambient” treatment. Specifically, all ASVs with a coefficient of variation (CV) ≥ the mean CV + 1\*SD (0.9111981) were culled, yielding a final count of 159 ASVs to be run through DESEq2. The log2 fold-change of ASV abundances was calculated between the three coral stress treatments and the “Non-bleached + Ambient” controls to elucidate ASVs that were differentially abundant when corals were bleached and/or thermally stressed (Table S5).

A total of 29 differentially abundant (DA) bacterial ASVs (18.2%) were identified (p ≥ .05 after FDR adjustment, Table 1). These ASVs belonged to 3 bacterial classes: Alphaproteobacteria, Gammaproteobacteria, and Bacteroidia, with the majority (69%) of DA ASVs belonging to Gammaproteobacteria. Within Gammaproteobacteria, there was a significant enrichment of numerous abundant Alteromonadaceae and Pseudoalteromonadaceae ASVs in at least one of the 3 treatments (80% of all DA Gammaproteobacteria ASVs).

In all 3 treatments there was a significant enrichment in 2 *Pseudoalteromonas* ASVs (1224 and 1296), 1 *Alteromonas* ASV (734), and 1 unclassified Saprospiraceae ASV (322). All 3 treatments also showed an enrichment of a highly abundant *Alteromonas* ASV (2), although this was only deemed statistically significant in the “Bleached + Ambient” and “Non-bleached + Heated” treatments. There was a concomitant reduction in the a highly abundant unclassified Cryomorphaceae ASV (3) in all 3 treatments, although this was not found to be significant. There were 12 DA ASVs found only to be significant in the “Bleached + Ambient” including significant enrichment in members of the genera *Psuedoalteromonas* (ASVs 75 and 85), unclassified Saprospiraceae (ASV 101), and *Phaeocystidibacter* (ASV 480), and significant reduction in members of the genera *Litoricola* (ASVs 27 and 419) and unclassified Alteromonadaceae (ASVs 368, 1012, and 1023). The two thermally stressed treatments, “Non-bleached + Heated” and “Bleached + Heated”, generally demonstrated similar patterns in DA ASVs. Specifically, in these two treatments there was a significant enrichment in ASVs of the genera *Psuedoalteromonas* (ASVs 853 and 823), *Aestuariibacter* (ASV 48), Thalassotalea (ASV 649), and an unclaasified Flavobacteriaceae (ASV 277).

**Do We Want A Synthesis Figure?**

**DISCUSSION**

This experiment presents a comprehensive assessment of how thermal stress and/or bleaching, separately and in combination, induce altered DOM exudation in corals, and in turn how this influences reef bacterioplankton community structure. We found clear evidence of both quantitative and qualitative differences in bulk DOM release and composition in corals that were heated, bleached, or both in combination. The DOM pools exuded from heated and bleached corals yielded elevated bacterioplankton abundances and enriched distinct bacterioplankton communities that were, broadly speaking, enriched in fast growing/opportunistic copiotrophs and depleted in bacteria found on “healthy/pristine” reefs. These data suggest that corals that experience elevated temperatures and/or bleaching alter water column biogeochemistry and microbial dynamics with potential negative feedback mechanisms further hampering coral resistance to and recovery from the damaging effects of thermal stress. Importantly, it appears that the broad patterns of these deleterious effects are consistent regardless if a coral is actively bleaching, is bleached in elevated temperatures, or is bleached and no longer experiencing elevated temperatures. Indicating a) that thermal stress, even in bleaching resistant corals, can lead to these deleterious effects and b) that even after periods of thermal stress have occurred, corals that bleach may still negatively influence water column dynamics through the aforementioned mechanisms. These data shed light on new, potentially negative coral-water column feedback loops during periods of elevated temperatures and provide the basis for potential water column-based, early detection of coral thermal stress *in situ*.

**Heat and Bleaching Status Alter Coral DOM Exudation**

Coral reef benthic primary producers are known to exude measurable quantities of DOC, a reality which is further confirmed here [(Haas et al., 2011, 2013; Nakajima et al., 2018; Nelson et al., 2013)](https://www.zotero.org/google-docs/?SCiywr)**.** Despite marginal significance (likely due to low sample size and short exudation time), all four coral treatments showed increased concentrations of DOC compared with the water controls (Figure S1, Kruskal-Wallis chi-squared=9.3187, p=.09).

Within the four coral treatments there appeared to be differences in DOC release, although these differences were not significant (likely due to low replication due to DOC contamination) (Figure 2, Kruskal-Wallis chi-squared=4.1667, p=0.244). Specifically, “Non-bleached + Heated” and “Bleached + Ambient” coral exudates were about 300% and 200% more concentrated (DOC per unit surface area, respectively) than their healthy, “Non-bleached + Ambient” coral counterparts, while “Bleached + Heated” coral DOC exudate were substantially less concentrated, maintaining DOC levels equivalent to that of the background water. Metabolomics data revealed substantial qualitative differences between the various DOM pools. Within the coral DOM samples, the two heated treatments (“Non-bleached + Heated” and “Bleached + Heated”) had distinct DOM pools, whereas “Bleached + Ambient” and “Non-bleached + Ambient” samples maintained unique clusters.

These data suggest that DOM exudate concentration and composition exhibit non-concordant patterns with respect to treatment. In terms of concentration, DOC values varied between the two heated treatments and remained relatively constant between the two ambient treatments. However, DOM composition remained similar between the two heated treatments and were distinct from both “Bleached + Ambient” and “Non-bleached + Ambient” coral treatments. These seemingly disharmonious patterns can simply be explained by the fact that heat and bleaching stress affect coral exudate quantity and quality in different ways.

The largest increase in DOC is from “Non-bleached + Heated” corals - corals that can be viewed as actively bleaching since their symbiont concentration was lower than the “Non-bleached + Ambient” treatment. Much of coral DOM exudate is known to be derived from Symbiodiniaceae (REF). Photosynthetic enzymatic reactions performed by Symbiodiniaceae, the upstream reactions that supply corals with organic Carbon they can then exude, are sensitive to/increase with temperature. SENTENCE WITH SOMETHING ABOUT ENZYME KINETICS? OR EXPERIMENTAL EVIDENCE OF RXN RATES IN SYM? (REFS). Maintenance of intact coral-algal symbiosis in the face of elevated temperatures may therefore lead to noticeably higher DOC exudation, with DOM exudates being the products of elevated photosynthetic reactions. Multiple studies have observed elevated particulate organic carbon (POC) release by corals under heat treatments, [(Niggl et al., 2009; Tremblay et al., 2012)](https://www.zotero.org/google-docs/?Gm6JoK) with two potential mechanisms proposed: a) controlled release of mucus by the corals to combat water column pathogens and b) uncontrolled release of POC (mucus and Symbiodinium) due to photooxidative damage and Symbiodiniaceae death/ejection from elevated light and/or temperature [(Wooldridge, 2009)](https://www.zotero.org/google-docs/?83DFex). However, the current study specifically isolated the dissolved fraction via .22um filtration of coral incubations, thus excluding mucus and other POC sources. To our knowledge no other studies have observed differences in DOC release/uptake between healthy and heated and/or bleached corals [(S. Levas et al., 2015; S. J. Levas et al., 2013; Niggl et al., 2009)](https://www.zotero.org/google-docs/?0KvZKV). Rather than our results being an aberration, it is more likely that DOC values from other experiments were inaccurate. Specifically, coral derived DOC is known to be relatively labile to bacterial degradation on the order of hours/tens-of hours (nelson/haas refs), yet many of these experiments derive their DOC values from incubations done in whole seawater or for over 10 hours, in both cases allowing bacterioplankton enough time to consume exuded DOC, thus artificially lower DOC concentrations and not capturing the full extent of coral DOC release during bleaching.Even in the case of Niggl *et al.* who used pre-filtered water for DOC exudations, the corals were left in the exudation chambers for 12 hours, potentially allowing bacteria enough time to grow and degrade coral derived DOC. In heated corals that also had reduced Symbiodiniaceae densities due to bleaching (“Bleached + Heated”), DOC concentrations were much lower, yet DOM composition was similar. This indicates that in heated corals, DOC exudate concentration is a function of symbiont density, yet heating, regardless of symbiodinaceae density, influences DOM composition. [HILLEYER ET AL REFS.]

Bleached corals that were kept at ambient temperatures (“Bleached + Ambient”) yield DOC exudates with similar concentrations to healthy corals (“Non-bleached + Ambient”), yet compositionally their DOM pools are distinct from each other and that of heated corals. After exposure to thermal stress, bleached corals are known to catabolize internal carbon stores to meet their energetic demands that are no longer satisfied by photoautotrophy. This includes [LIST EXAMPLES]. It is possible that DOM mobilization from internal storage could yield similar concentrations of DOC exudate compared to healthy corals, despite reduced levels of C fixation. However, the catabolization of internal lipids, etc. via distinct metabolic pathways is quite likely to yield DOM exudates qualitatively distinct from that of non-bleached corals, an observation found in this study.

**DOM exudates from stressed corals alter bacterial growth**

Different DOM exudates yielded different levels of bacterial abundance and growth rates. In general, DOM derived from coral treatments yielded higher microbial abundances and growth rates than the water controls. Within the coral treatments, any form of holobiont “stress” (whether bleaching, heating, or the combination) yielded higher bacterial abundances than the healthy, “Non-bleached + Ambient” controls. Specifically, DOM from “Non-bleached + Heated” corals yielded higher microbial growth rates and significantly higher bacterial concentrations than the control DOM treatment (Tukey post-hoc test, p<.05), with bacteria grown on this DOM reaching concentrations nearly double that of the control DOM treatment. It is likely that the increased DOC concentration from”Non-bleached + Heated” corals engendered the rapid growth of bacterial communities to significantly higher concentrations than healthy corals and water controls. This rapid growth to high bacterial abundances indicates that DOC released from stressed corals is labile - readily mobilized by bacterioplankton to fuel growth via respiration and into anabolic pathways (REF).

Despite the lowest DOC concentrations, the combined “Bleached + Heated” treatment still had higher bacterial abundances and growth rate than the “Non-bleached + Ambient” control. The DOM composition of this treatment was identical to the “Non-bleached + Heated” treatment, indicating that the increased microbial growth is not just a function of DOM quantity, but quality as well, and that elevated temperatures induce coral, regardless of their bleaching status, to exude DOM that is rapidly assimilated by bacterioplankton communities. Rapid growth of bacterial communities to high concentrations could harm corals through a variety of mechanisms including the generation of hypoxic zones from high levels of bacterial respiration. LIST EXAMPLES.

**Ecological Implications**

To understand the ecological implications of bleaching on DOM release and the microbial community we can use the current study to assess the different stages during a mass bleaching event. Where the “Non-bleached + Ambient” treatment represents healthy coral reefs. The “Non-bleached + heated” treatment is representable for the period where the corals are actively bleaching, losing their symbionts. After that period, corals are bleached and it is highly likely corals will first experience a period of elevated temperatures, represented by the “Bleached + Heated” treatment. Once temperatures go down, corals will remain bleached for a while (“Bleached + Ambient” treatment) before - hopefully - recovering their symbiont levels. In this study we see that actively bleaching and bleached corals release DOM that supports and enriches the growth of bacterial lineages associated with copiotrophic life strategie in dilution cultures. One can imagine that at the start of a bleaching event on a healthy reef, there is a stable “healthy” microbial community. For this community to shift to a copiotrophic community, there is a need for a lot of energy. However, given the increased released DOC of actively bleaching corals, and the change in DOM composition that remains a food source for these copiotrophic communities the bleached treatments, we hypothesize that a mass bleaching event, given that enough corals bleach in the same short period, supplies enough energy in the form of labile carbon that could lead to a reef wide shift in microbial community composition. This study did not take into account increased temperatures during microbial growth, which would also increase metabolic rates of the microbial community, which could be an additional factor for a rapid switch to copiotrophic community. A similar shift to copiotrophic lineages is observed on algae dominated reefs, where the microbial community has shifted to higher microbial biomass and energy use, also known as microbialization [(Haas et al., 2016)](https://www.zotero.org/google-docs/?6HqnWi)**.** Microbializtion is part of the DDAM model [rohwer papers], which is a negative feedback loop where increased algae abundance, increases and changes DOM release fostering a more copiotrophic microbial community which in their turn has a negative effect on the coral cover leaving room for the algae to expand and release more algal DOM. Taking the DDAM model in account, a shift in the watercolumn microbial community might make it more difficult for corals to recover than when the microbial community is still resembling a “helathy” coral reef for instance during a short bleaching event caused by short high light intensities and/or temperature peaks instead due to prolonged accumulation of heat stress, like the moorea 2019 mass bleaching event. It is also known that during a massive bleaching event, part of the corals will not survive, leaving open substrate to be rapidly occupied with fast growing (macro-)algae. Shifting back to a microbial community that resembles a “healthy” reef and with that having a fully recovered system, might take longer than corals regaining their symbionts, leaving the system more vulnerable than meets the eye.

**Conclusion**

Assessing the DOM release by corals mimicking the different stages during a bleaching event (healthy, bleaching, bleached still in elevated temperatures, and recovering) effects everything

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