Coral Bleaching Induced Mortality Transforms Local and Global Carbon Cycles: A Positive Feedback Loop That May Accelerate Reef Decline

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Supplementary Methods

Study Site:

All parts of this study were conducted at the Mo'orea Coral Reef Long-Term Ecological Research (MCR-LTER) station on the island of Mo'orea in French Polynesia (-17.475, -149.837) on the forereef on the north shore of the island, using LTER sites between 5-10m depth with high coral cover, and all near-reef and off-reef samples following the steep slope out from the reef to 1 km where depths exceed 500m.

Field Sampling for Bleaching Event:

Water was collected during the initial onset of the bleaching event on April 1, 2019, before temperatures peaked. Water chemistry was then sampled following the most intense temperature peaks (April 28 - May 15, 2019). Collections occurred at 1m, 5m, and 10m depths over a 10m deep site on the forereef that has been sampled for over a decade as part of the LTER station time series sampling (LTER-1). Samples were taken at three points along a transect that ran parallel to the reef, separated by 15m. Upon collection, samples were stored on ice until processing in the lab, with no more than 2 hours elapsing between sampling and processing.

Quantification of Bleaching extent:

Images of the Mo'orea fore reef that included scale bars were annotated using ImageJ as either bleached (completely white), partially bleached (contained some pigment but had clear areas of bleaching), or unlikely to be bleaching (no visible white spots beyond normal for the species). The surface area of each was quantified through tracing the exterior of individual corals and

summing across the plots which were between 46 and 93 m^2 apiece with three areas of the forereef measured, corresponding to MCR LTER sites 0, 1, and 2. The resolution of the images was such that individual species could be easily identified.

Mesocosm Experimental Design and Sampling:

Corals were collected at 5m depth on the forereef at 3 sites (upslope from LTER sites: LTER0, LTER1, and LTER2). A coral colony was collected (as a whole) via chisel and hammer, placed in a plastic bag and transported in a cooler back to the research station. The colony was then fragmented into ca. 2cm x 2cm fragments and allowed 24 hours acclimation time in a running seawater table in partial shade. During the evening, hypoxia was avoided by lightly bubbling the tanks and keeping a low density of corals per container. After acclimation, corals were placed in individual closed systems (the 'mesocosm') which consisted of a 500ml glass jar (PTFE lined lid) that was connected to mylar bags (1.0 L) and circulated with a peristaltic pump continuously throughout the experiment. This design allowed for a large enough water reservoir to avoid nighttime hypoxia, while also allowing enough sunlight exposure to facilitate natural cycles of photosynthesis. These systems were cleaned between replicates with acid washing, a methanol rinse, and a final rinse and soaking with MilliQ water. Each mesocosm was filled with filtered seawater (FSW; 0.2 μm). Corals were exposed to 3 treatments: no modification, +3°C temperature increase (controlled with thermistors and quantified by ONSET Hobo temperature recorders), and coral exudate. The coral exudate was created by forcing bleaching of coral fragments (from the same colony that was used for the given replicate) in sterile containers filled with FSW via exposure to temperature and light stress for 24 hours. After separating the filtrate from other material by passing it through a coarse, non-quantitative filter to remove particulates and mucus aggregations, we diluted the concentrated exudate and added 0.5 L of the exudate to each treatment.

DOC concentration measurement

Sample processing

Water samples for DOC analysis from both field sampling and the mesocosm experiment were filtered through pre-combusted (450°C for 5 hr) 25mm GFF filters, with the filtrate stored in

acid-leached, MilliQ flushed, sample rinsed 60 mL polyethylene bottles at <-20°C. DOC samples were sent to the Nutrient Analytical Services at the Chesapeake Biological Laboratory of the University of Maryland Center for Environmental Science.

Samples were acidified and sparged with ultra-pure carrier-grade air to drive off inorganic carbon and analyzed using the high-temperature combustion (680°C) method on a Shimadzu TOC-L following operating procedures outlined in (Total and Dissolved Organic Carbon Method: https://www.umces.edu/nasl/methods). In short, all carbon compounds are broken down into CO_2 which is carried by ultra-pure air to a non-dispersive infrared detector (NDIR) for detection. The system was calibrated before and after each run with potassium hydrogen phthalate standards (6-point curve, 0-20 mg C/L) and referenced against a certified reference control sample (Scp Science Accuspec Toc Standard), a spike (20 ppm) and a blank every 10 samples. The acceptance criteria used was 0.995.

Symbiodiniaceae photosynthetic efficiency

The Fv/Fm, or photosynthetic efficiency, of Symbiodiniaceae cells within dark-adapted coral fragments was measured using a diving Pulse Amplitude Modulated (PAM) Fluorometer (Walz Diving PAM) at five time points per experimental replicate (t=0, 6, 12, 24, and 48 hours). Corals were placed in 0.2 µm FSW within dark-adaptation boxes for 20 minutes prior to measurements to ensure that the Photosystem II of Symbiodiniaceae cells had entered a relaxed state. At each T0, four control fragments and one temperature and one coral exudate fragment were measured (n = 6 total). Three of these control fragments were subsequently sacrificed for isolation of genomic material and measurement of Symbiodiniaceae densities and chlorophyll concentrations (see subsequent sections). At all time points, one fragment per treatment (control, temperature and coral exudate, n = 3 total) was measured and then sampled for downstream analyses.

PAM fluorometry measurements were conducted in the dark using the following instrument settings: pulse width of 0.8s, light intensity of 12, signal dampening of 2, and a gain of 5. The "measure light intensity" parameter was set to 3. The PAM fluorometer was auto-zeroed in darkness, and then measurements were taken with the beam probe at 5 mm from each coral surface. Three separate fluorometry measurements were collected per coral fragment, each on a

different area of the coral. Changes in Fv/Fm were analyzed using a 1-way ANOVA with Tukey Posthoc test with time as the independent factor in SigmaPlot 11.

Categorical Coral Health

In addition to the below referenced quantitative measures of bleaching, we used an observational catagorical evaluation of the level of bleaching undergone by the corals based on imagery. We used a course scale from 1-5: (5) Coral appears healthy with no obvious signs of bleaching; (4) Coral appears partially lighter in color than T_0 control corals; (3) Areas of blotchy tissue and/or obvious partial bleaching of part of the coral; (2) Coral is completely bleached; (1) Coral tissue has begun to slough off indicating a dead coral individual. These rankings were based on the images (images provided in Fig. S5) and were used as a metric of bleaching due to the inherent and high variability across replicates which sometimes obscured visually obvious patterns of coral health. Significance of these patterns was analyzed through a 1-way ANOVA with time as the independent variable. A tukey posthoc test was used in the case of a significant result.

Symbiodiniaceae chlorophyll concentration and cell densities

Sacrificed coral fragments were stored at -40°C. After thawing, an airbrush supplied with 0.02 µm FSW was used to blast coral tissue into a sterile 200 ml whirlpak. The blastate was then homogenized using a Fisherbrand 150 handheld homogenizer (Waltham, Massachusetts) for ten seconds at full speed. To measure chlorophyll concentrations, four 1 mL aliquots of homogenized blastate were transferred to 1.5 ml centrifuge tubes, and centrifuged for 3 min at 15,000g to form a concentrated symbiont pellet that was immediately frozen at -40°C. Pellets were subsequently resuspended in 1 ml of 90% acetone, sonicated at 40% amplitude, and placed in the dark. After 24 hours, 250µl of this solution was loaded in triplicate into a 96-well plate and absorbance at 630nm, 647nm, and 664nm was recorded. The concentrations of chlorophyll a and chlorophyll c2 were calculated using equations from (Jeffrey & Humphrey, 1975) New spectrophotometric equations for determining chlorophyll a, b, c1 and c2 in higher plants, algae and natural phytoplankton and normalized to fragment surface area.

To quantify Symbiodiniaceae cell densities, two 250µl aliquots of the homogenized blastate (see above) were transferred to 2 ml cryotubes with 250µl of 10% formaldehyde in 0.02 µm-filtered

seawater and stored at 4°C. The remaining volume of blastate was measured using a 2 ml serological pipette; total volume of blastate was used to calibrate chlorophyll concentrations and cell densities with coral fragment surface area. Symbiodiniaceae cells were counted using light microscopy (20X) and a Neubauer hemocytometer. Cell concentrations were counted in sixteen replicate squares (N=4 technical replicates/sample) and the concentration per ml blastate was normalized to fragment surface area.

Coral fragment surface area

After tissue blasting, a mean of 75 pictures were taken of each coral fragment using an iPhone 6 at a 10° and 60° angle. The pictures were loaded into Agisoft Metashape (St. Petersburg, Russia) and processed using the following settings: Photos were aligned using generic preselection at the highest accuracy setting, excess points were then removed manually, a dense cloud was constructed using the high accuracy option, a mesh was constructed using 'dense cloud, high face count' as the source data, and a texture was applied using the 'Generic - Mosaic' setting. Four markers were then placed in the scene at known distances, and three scale bars were added between them. Any remaining model area that was not part of the coral fragment was manually removed, and surface areas were measured using the 'Measure area and volume' option.

Symbiodiniaceae genetic diversity

Coral tissue samples were preserved in DNA/RNA shield and DNA was extracted using the ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research, Irvine, CA) and sequenced on the Illumina MiSeq Platform using Symbiodiniaceae Internal Transcribed Spacer-2 (ITS-2) specific primers (Sym_VAR_5.8SII and Sym_VAR_REV, 32). The PCR reaction contained 5µl of DNA (5ng/µl), 2.5µl of SYM_VAR_5.8SII + MiSeq Adapter, 2.5µl of SYM_VAR_REV + MiSeq Adapter (2 µM), 12.5µl 2x KAPA HiFi HotStart ReadyMix, and 2.5µl of molecular grade water for a total reaction volume of 25µl. PCR cycles were as follows: 95°C for 3 min, 15 cycles of 95°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec, and 72°C for 4 min. PCR clean-up was completed using Agencourt AMPure XP Magnetic Beads. Illumina indexing primers were added to 50µl of purified PCR product, and a new PCR was run to incorporate unique barcodes. The PCR reaction contained 5µl of cleaned PCR product, 5µl of Illumina Indexed Primer 1 (i5), 5µl

of Illumina Indexed Primer 2 (i7), 25µl 2x KAPA HiFi HotStart, and 10µl PCR Grade water for a total reaction volume of 50µl. PCR cycles were as follows: 95°C for 3 min, 20 cycles of 95°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec, and lastly 72°C for 4 min. The resulting PCR product was purified with Agencourt AMPure XP Magnetic Beads. Samples were quantified via qPCR using the KAPA library quantification kit and normalized and pooled in equimolar amounts. Pooled samples were sequenced on the Illumina MiSeq platform using a PE300 run with 25% PhiX at the Georgia Genomics and Bioinformatics Core (University of Georgia, Athens, GA).

Demultiplexed fastq files were generated with Illumina's BaseSpaceFS (version 1.5.964) and reads were processed in RStudio (version 1.1.456) using the DADA2 pipeline (version 1.11.0, Callahan *et al*. 2016.) with modifications for the Symbiodiniaceae ITS-2 region. Samples with fewer than 10,000 reads (n = 2) were removed from the dataset. The DADA2 pipeline generated a table of amplicon sequence variants (ASVs) which was then curated via the LULU pipeline, which uses co-occurrence patterns and sequence similarity to eliminate erroneous ASVs (Frøslev *et al.*, 2017). Symbiodiniaceae ITS-2 types were then assigned based on BLAST results to a local Symbiodiniaceae ITS-2 database (Cunning *et al.*, 2017).

Data Visualization

Contour plots of DOC concentrations were made in Ocean data viewer (v.5.1.7) using the DIVA-gridding algorithm and interpolating to 30x30 scale-length grid, which has been indicated as reliable for avoiding over-interpolation. Comparisons were made to data collected as part of the MCR-LTER time series sampling (and data replotted from Nelson *et al.*, 2011). These later data are measurements of total organic carbon (TOC), of which the particulate pool is a small proportion of (3-5%) and differences between reef and offshore concentrations have been shown to not be significantly driven by the particulate pool. Therefore, we treat these TOC values as DOC as has been done previously (Nelson *et al.*, 2011). Data from the mesocosm experiments were averaged across replicates per species to give a representative view of behavior of each coral species throughout the experiment. Responses relative to specific replicates are given in SI Figure 4. We used ggplot2 in R to produce the base heatmaps, boxplots, and remaining data visualization.

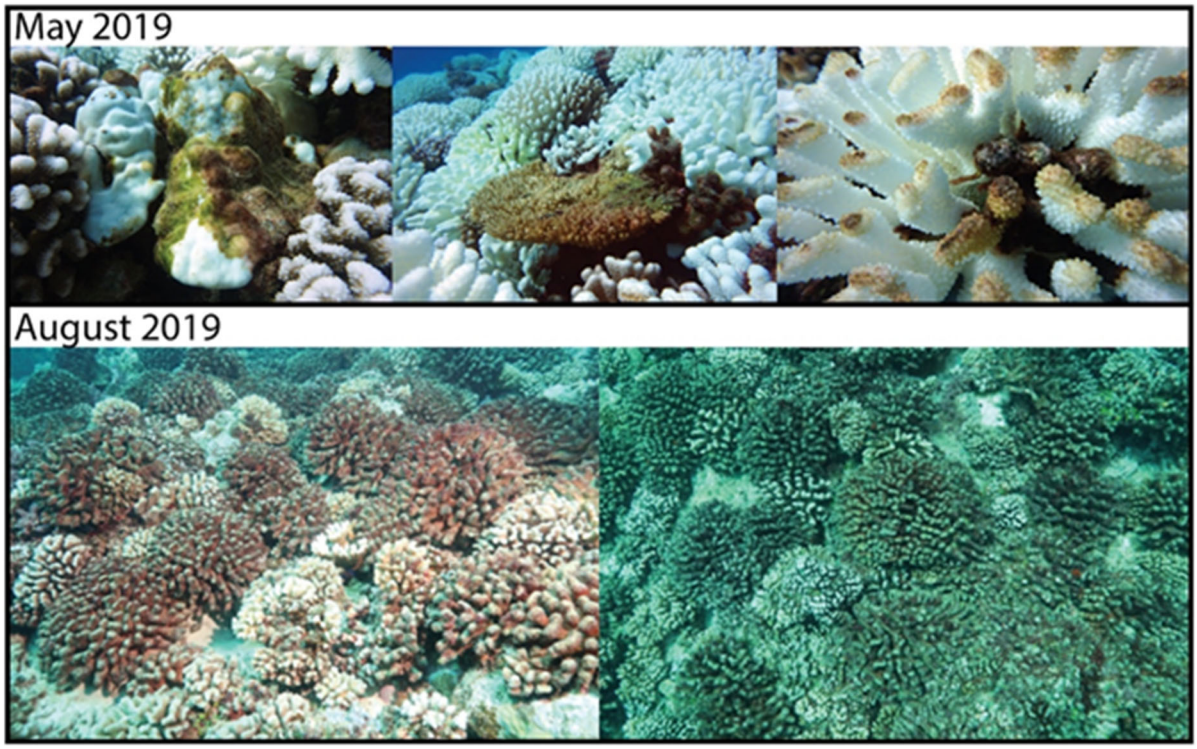
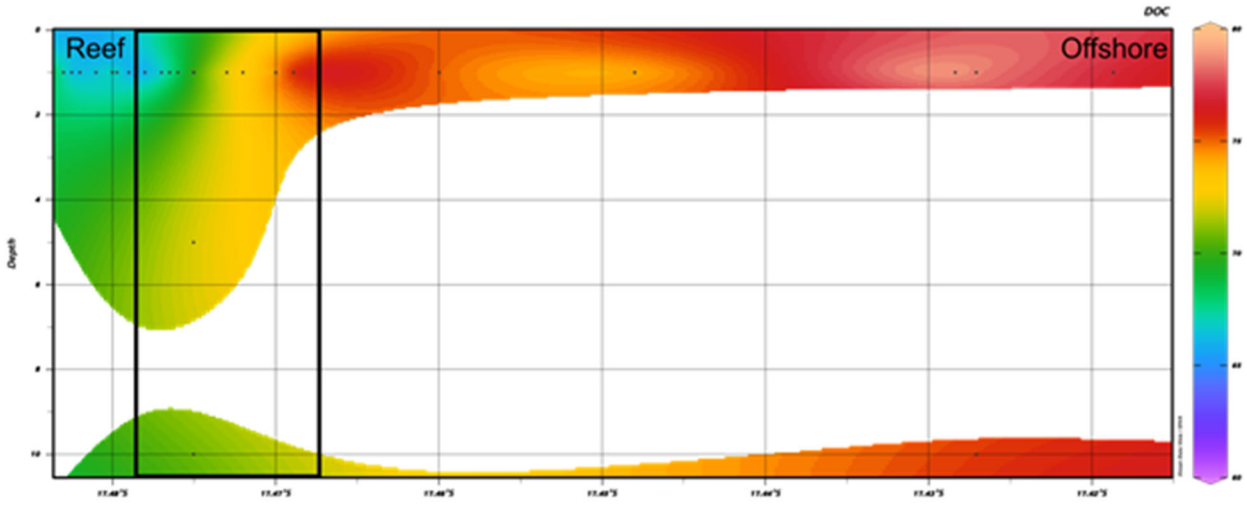


Fig. S1: The progression of macroalgal overgrowth following the April 2019 bleaching event, from patchy areas on distinct colonies (May 2019) to whole colonies across the reef-scape (August 2019). In May of 2019, significant portions of bleached colonies remained alive (stark white areas). Light to dark brown areas had died and were overgrown by macroalgae. By August of 2019, many colonies had experienced full mortality and were completely overgrown by macroalgae (brown colonies).



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178 **Fig. S2:** Full range of DOC data for the combined data set, going out 5km from the reef crest.
179 Black box indicates the range plotted in Figure 1.

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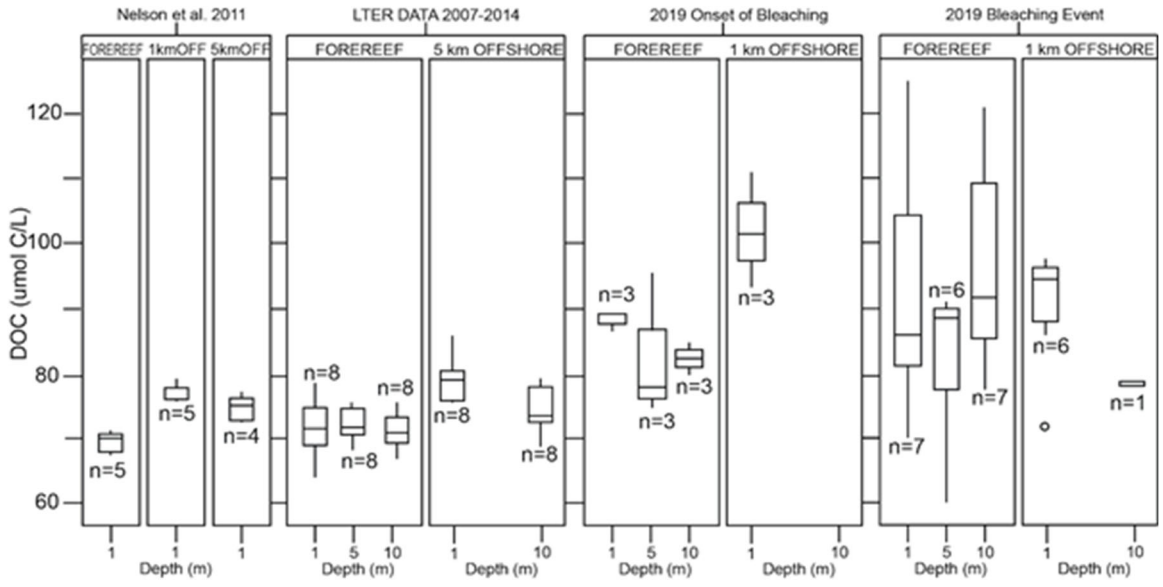

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182 **Fig. S3:** DOC concentrations across the datasets used for forereef and offshore gradients. The
183 number of samples within each group is indicated.

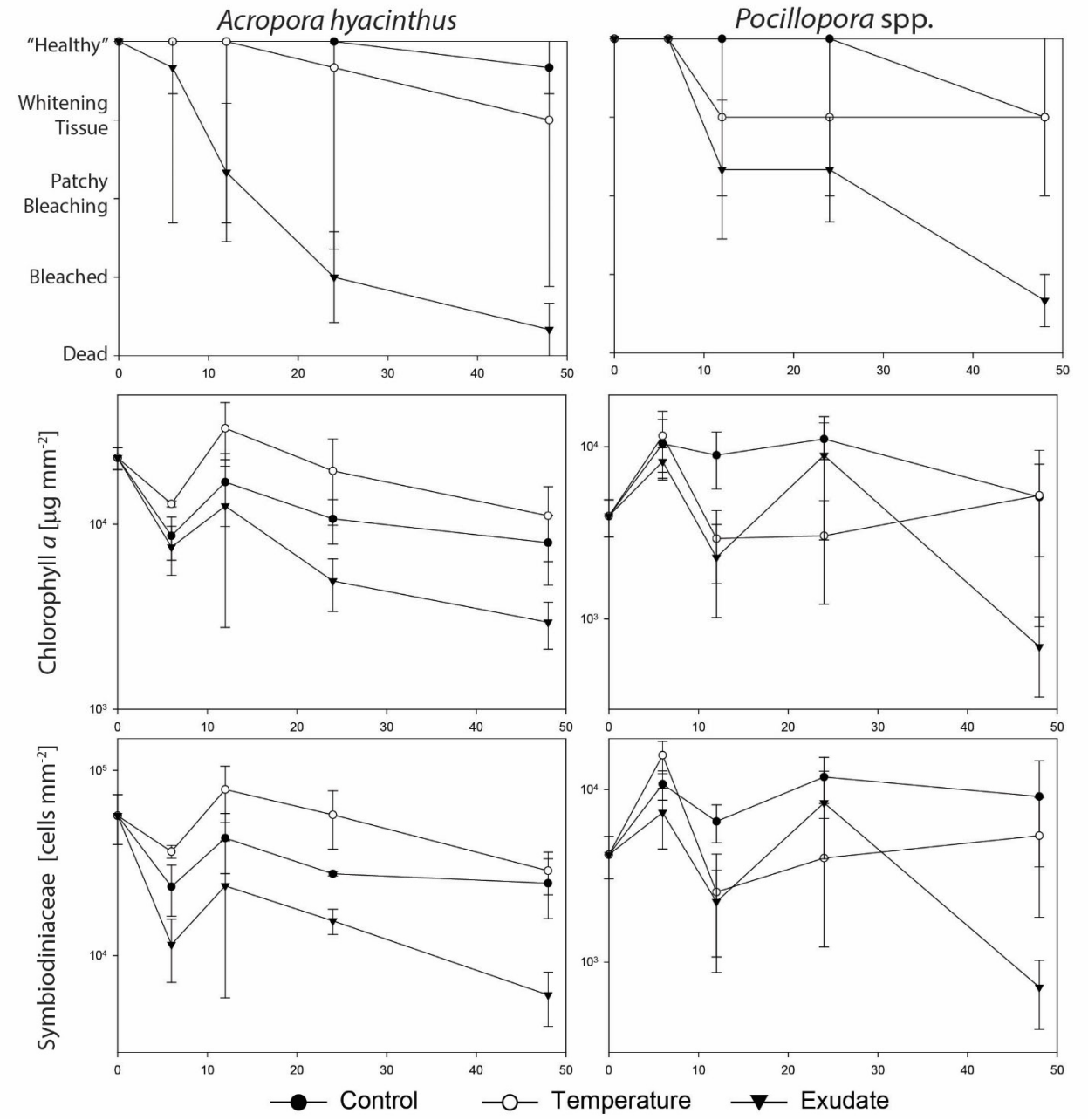
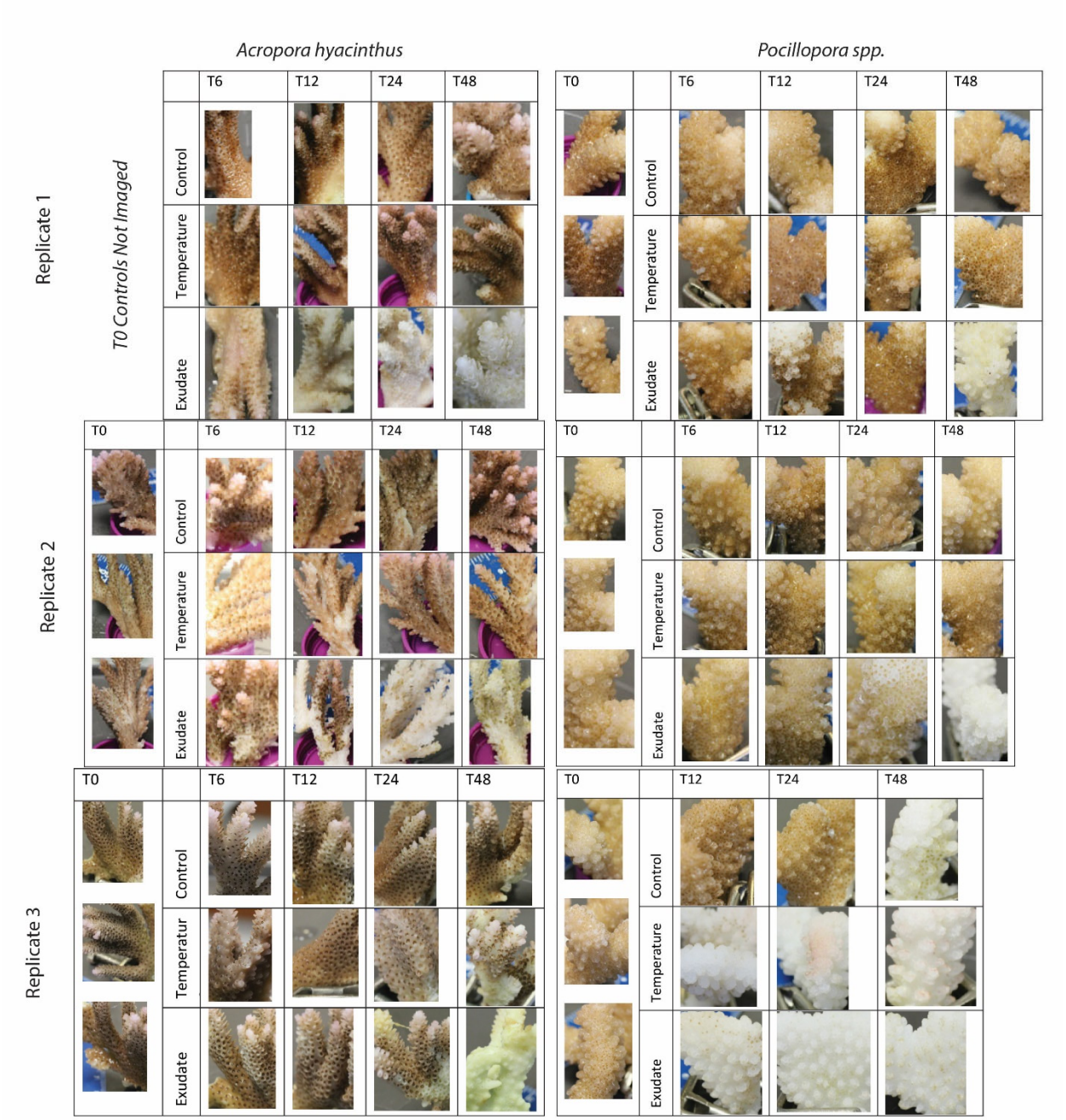


Fig. S4: Impact of experiments on coral bleaching state (top), concentration of Chlorophyll a within the coral holobiont tissues (middle), and density of Symbiodiniaceae (bottom) in *A. hyacinthus* (left) and *Pocillopora* spp. (right). Error bars are Standard Error except for T6 for *Pocillopora* spp. where only 2 replicates were carried out, and error bars are the range. Coral health used a 1-5 scale based on consensus opinion observations from no apparent divergence from T0 control to increasingly bleached with "dead" defined as tissue sloughed off. For comparison, images of each individual for each treatment are provided in Fig. S5.



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193 **Fig. S5.** Impact of temperature and exudate stress on two species of corals as displayed by
194 imagery of each of the individual nubbins sampled for each treatment.

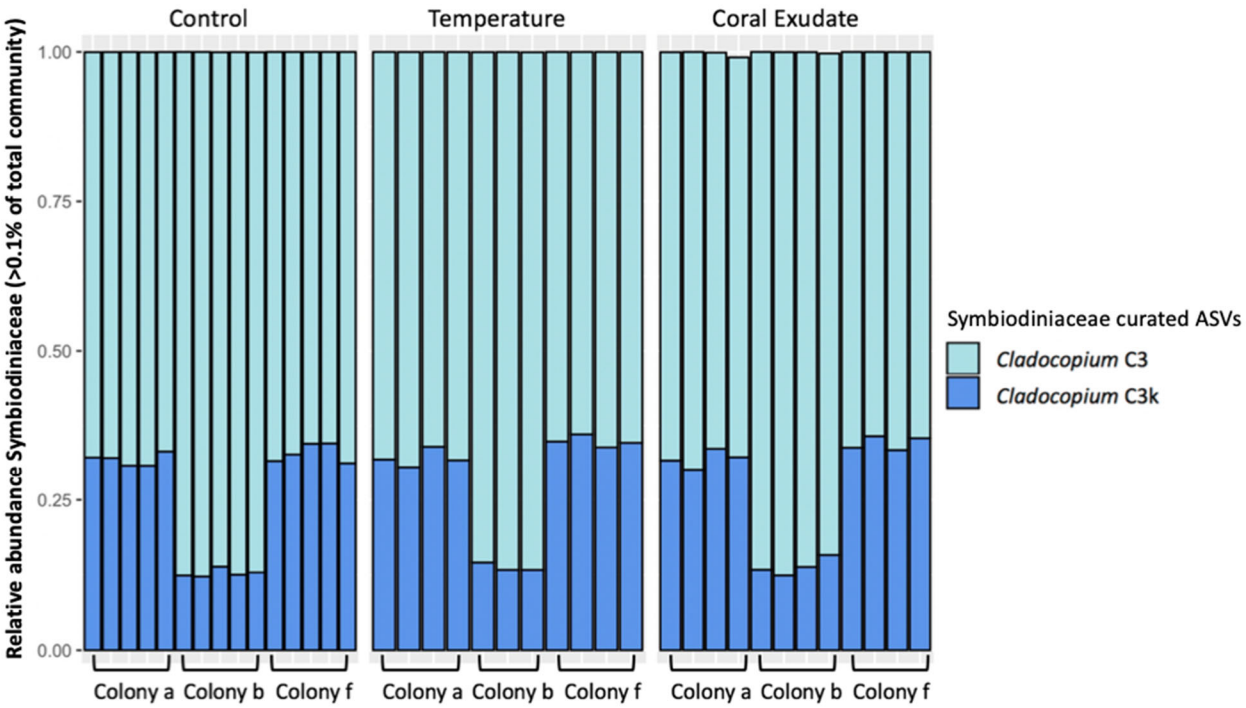

Fig. S6: Relative abundance of the dominant (>0.1% of total community) Symbiodiniaceae curated amplicon sequence variants (ASVs) based on the internal transcribed spacer-2 (ITS-2) region of rDNA in fragments of three *Acropora hyacinthus* genets exposed to high temperature (+3˚C), coral exudate (+ ~2mM DOC), or ambient (control, ~27˚C) conditions. The identities of curated Symbiodiniaceae ASVs were assigned based on BLAST results to a local Symbiodiniaceae ITS-2 database (Cunning et al., 2017): *Cladocopium* C3 (NCBI Accession #: AB778606), *Cladocopium* C3k (NCBI Accession #: AY589737). Each coral fragment is interpreted as containing a single Symbiodiniaceae species in the genus *Cladocopium*; within this species, the *Cladocopium* C3 and C3k curated ASVs are interpreted as intragenomic variants (Davies et al. *in review*). Fragments within the same colony and treatment were sampled at up to five timepoints over a 48-hour period, and are ordered here from earliest to latest per genet.

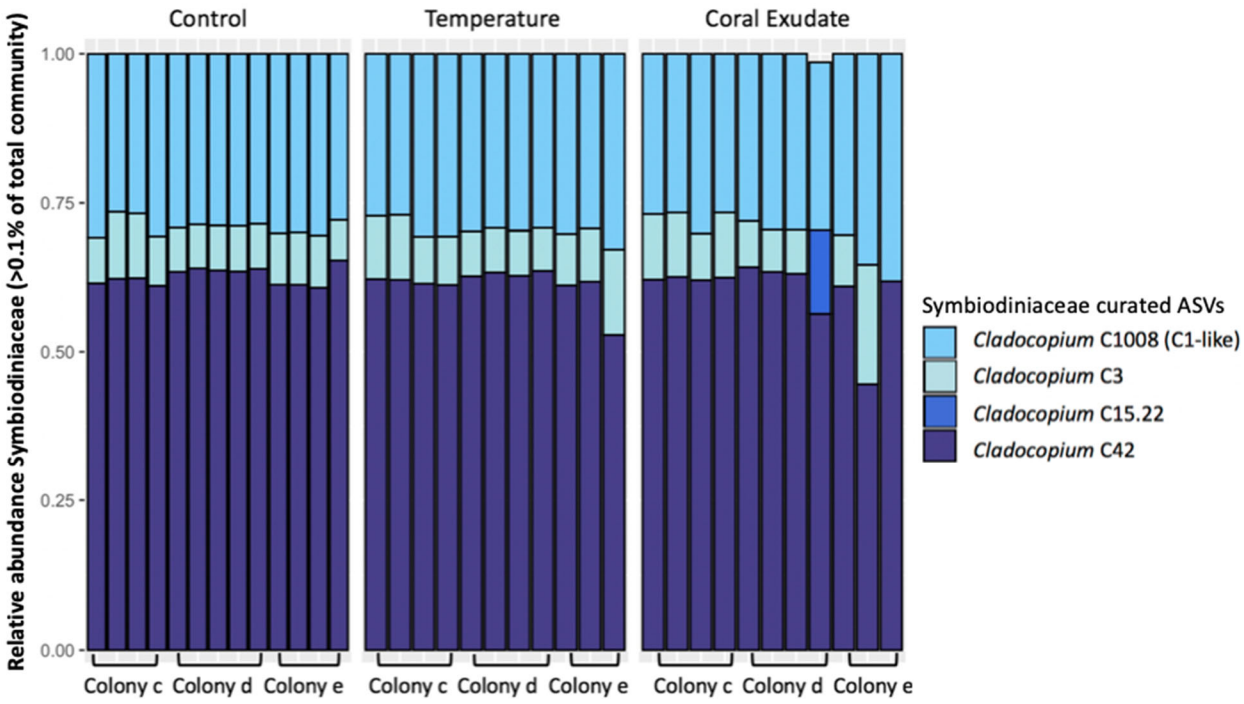

Fig. S7: Relative abundance of the dominant (>0.1% of total community) Symbiodiniaceae curated amplicon sequence variants (ASVs) based on the internal transcribed spacer-2 (ITS-2) region of rDNA in fragments of three *Pocillopora* genotypes exposed to high temperature (+3˚C), coral exudate (+ ~2mM DOC), or ambient (control, ~27˚C) conditions. The identities of curated Symbiodiniaceae ASVs were assigned based on BLAST results to a local Symbiodiniaceae ITS-2 database (Cunning et al., 2017): *Cladocopium* C1008 (C1-like, Cunning et al., 2015, NCBI Accession #: DQ480613), *Cladocopium* C3 (NCBI Accession #: AB778606), *Cladocopium* C15.22 (NCBI Accession #: KC597695), *Cladocopium* C42 (NCBI Accession #: AY765402). Coral fragments are interpreted as containing a single Symbiodiniaceae species in the genus *Cladocopium*; curated ASVs within each sample are likely intragenomic variants (Davies et al. *in review*). The rightmost fragment of colony d in the coral exudate treatment may contain a different *Cladocopium* symbiont than the rest of the fragments, but this cannot be fully resolved from the sequence data available. Fragments within the same colony and treatment were sampled at up to five-time points over a 48-hour period, and are ordered here from earliest to latest per genet.

Table S1: ANOVA results identifying difference in Coral Health Categorical Variables, Symbiodiniaceae (Symbiont) density, and Chlorophyll *a* Density as impacted by the various treatments over time. Significant 2 way interactions meant that a 2-way ANOVA was not informative and so 1 way ANOVAs were run with Tukey post-hoc tests across time. Degrees Freedom varied among tests due to time points missing the required 3 replicates for an informative test. Statistics were run in SigmaPlot 11 and '=' in the post-hoc results indicates no significant difference, not necessarily equivalence.

Fv/Fm	*Statistic*	*Post-hoc results*
Pocillopora spp.		
Control	$F_{4,36}$=0.8, p=0.554	Non-significant test
Temperature	$F_{4,15}$=0.8, p=0.560	Non-significant test
Exudate	$F_{4,15}$=5.8, p=0.005	$[T_0 = T_6] \neq T_{48}$
Acropora hyacinthus		
Control	$F_{4,26}$=2.2, p=0.100	Non-significant test
Temperature	$F_{4,16}$=0.6, p=0.640	Non-significant test
Exudate	$F_{4,16}$=12.7, p<0.001	$[T_0 = T_6] \neq [T_{12}=T_{24}=T_{48}]$ except $T_6=T_{24}$
Categorical Health		
Pocillopora spp.		
Control	$F_{3,14}$=1.9, p=0.169	Non-significant test
Temperature	$F_{3,14}$=1.2, p=0.357	Non-significant test
Exudate	$F_{3,14}$=16.2, p<0.001	All pairwise comparisons significant
Acropora hyacinthus		
Control	$F_{3,13}$=1.4, p=0.303	Non-significant test
Temperature	$F_{3,13}$=3.0, p=0.06	Non-significant test
Exudate	$F_{3,13}$=15.6, p<0.001	$[T_0 = T_6] \neq [T_{24}=T_{48}] \& T_0 \neq T_{12} \& T_6=T_{12}$
Symbiont Density		
Pocillopora spp.		
Control	$F_{3,13}$=1.8, p=0.203	Non-significant test
Temperature	$F_{3,13}$=0.4, p=0.868	Non-significant test
Exudate	$F_{3,13}$=2.1, p=0.156	Non-significant test
Acropora hyacinthus		
Control	$F_{4,15}$=1.2, p=0.344	Non-significant test
Temperature	$F_{4,15}$=0.8, p=0.522	Non-significant test
Exudate	$F_{3,13}$=2.4, p=0.118	Non-significant test
Chlorophyll a		
Pocillopora spp.		
Control	$F_{3,13}$=2.8, p=0.090	Non-significant test
Temperature	$F_{3,13}$=0.2, p=0.881	Non-significant test
Exudate	$F_{3,13}$=1.6, p=0.235	Non-significant test
Acropora hyacinthus		
Control	$F_{4,16}$=3.0, p=0.050	No significant Post-hoc ($T_0=T_{48}$@p=0.11)
Temperature	$F_{3,13}$=1.2, p=0.32	Non-significant test
Exudate	$F_{3,13}$=4.0, p=0.031	No significant Post-hoc ($T_0=T_{48}$@p=0.08)

Table S2: DOC concentrations during bleaching experiment with three dominant coral types collected in Mo'orea, French Polynesia. Healthy corals were collected during May 09-13, 2019 and experiments were run until bleaching was observed (24-48 hours).

	Control DOC (mmol C/cm2 ± SE)	Bleached DOC (mM C/cm2 ± SE)
Pocillopora spp.	0.44±0.17 (n=3)	1.74±0.24 (n=5)
Pocillopora eydouxi	0.40±0.02 (n=2)	3.13±1.00 (n=3)
Porites lobata	0.13±0.01 (n=3)	2.52±0.45 (n=2)
No Coral Blank (mM C)	0.32±0.04 (n=3)	
AVERAGE	0.31±0.07 (n=8)	2.3±0.35 (n=10)

Table S3: DOC release by non-bleaching corals

Coral Type	Citation	um/dm/day	mmol/m/day	mmol/cm^2/day
Pocillopora	Haas et al 2013		1.32	1.32E-04
Mean (multiple coral types incl.)	Naumann et al 2012		2.2	2.20E-04
Porties	Haas et al 2011	55.2	0.0552	5.52E-04
AVERAGE				3.01E-04

Table S4: Reef surface area values

Reef Surface Area			
	km2	cm2	10%
Great Barrier Reef (Harris et al 2013)	20,678	2.07E+14	
Current Coral Coverage (Costanza et al. 2014)	280,000	2.8E+15	2.8E+14

Equation 1: Calculations for amount of DOC released in non-bleaching conditions

$$3.01e^{-4}\frac{mmolC}{cm^2}\ x\ 2.8e^{15}cm^2 = 8.44e^{11}\ mmolC = 8.44e^{8}molC$$

Equation 2: Calculations for amount of DOC released if 10% of corals bleach

$$2.3\frac{mmolC}{cm^2}\ x\ 2.8e^{14}cm^2 = 6.44e^{14}\ mmolC = 6.44e^{11}molC$$

Table S5: Determination of percentage of area bleached during 2016-2017 bleaching event based on Hughes et al., 2017

Great Barrier Reef Bleaching 2016				
Percent Bleached	1%	10%	30%	60%
20% SA Bin	0.00	0.02	0.06	
40% SA Bin				0.24
Sum of Bins				0.32
SA Bleached	0.01	0.10	0.30	0.60
20% SA Bin	4.14E+11	4.14E+12	1.24E+13	
40% SA Bin				4.96E+13
Sum of Bins (cm^2 bleached)				6.66E+13

Equation 3: DOC Release from 2016-2017 bleaching event

$$2.3\frac{mmolC}{cm^2}\ x\ 6.66e^{13}cm^2 = 1.53e^{14}\ mmolC = 1.53e^{11}molC$$

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