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PRACTICAL ARTICLE

Symbiodiniaceae probiotics for use in bleaching recovery

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Coral reefs are currently under threat as a consequence of local and global stressors, in particular, mass coral bleaching induced by climate warming. In conjunction with global cuts to carbon emissions, active restoration interventions are being investigated as an additional option to buy time while these stressors are mitigated. One intervention with the potential to improve recovery during or postbleaching involves the addition of probiotic treatments, that is the addition of microorganisms that provide benefits to the host. Fragments of the branching coral, *Acropora millepora*, were experimentally exposed to a bleaching event coupled with the inoculation of Symbiodiniaceae probiotics (*Durusdinium trenchii* and *Cladocopium goreaui*) to determine if these probiotic treatments could ameliorate bleaching related stress and mortality. Fragments inoculated with *C. goreaui* and exposed to 32°C for 6 days exhibited significantly less mortality (9.1 ± 5%) compared to corals exposed to 32°C without probiotics (66.7 ± 8%) or with *D. trenchii* (41.7 ± 9%). Fragments in the *C. goreaui* probiotic treatment also bleached less and exhibited the highest photosynthetic efficiency compared to fragments inoculated with the *D. trenchii* at 32°C. Internal transcribed spacer-2 amplicon sequencing did not detect the inoculated *D. trenchii* and *C. goreaui* cells within *A. millepora* tissues at the end of the experiment, suggesting the corals did not reestablish symbiosis but instead used inoculated cells as a nutritional supplement, although other factors such as shuffling conditions may have had an effect. This study highlights that nutritional supplementation can possibly aid coral resilience to temperature stress, though a far more detailed understanding of the factors that influence host regulation during symbiosis establishment is required.

Key words: bleaching, heat stress, probiotics, Symbiodiniaceae, symbiosis

Implications for Practice

- The addition of Symbiodiniaceae probiotics supports improved coral survival during heat stress, thereby improving projected outcomes for protection and restoration.
- The use of probiotics to induce manipulative shifts to more heat-tolerant Symbiodiniaceae communities appears limited.
- The use of Symbiodiniaceae probiotics for bleaching and bleaching-related mortality prevention and amelioration is a potential new restoration technique but will require substantial research effort to operationalize.

Introduction

The impacts of climate change on coral reefs are increasing in frequency and severity, driving corals closer to their thermal physiological thresholds and leading to mass die-offs on a global scale (Hughes et al. 2003; Hoegh-Guldberg et al. 2007; Riegl et al. 2009; Hughes et al. 2017). This increase in water temperature disrupts the relationship between the coral host and their symbiotic dinoflagellates of the Family Symbiodiniaceae, resulting in mass mortality if temperatures are sustained (Hoegh-Guldberg 1999; Baker 2003). The development of novel restoration interventions is becoming increasingly important to help promote recovery and resilience of corals (van Oppen et al. 2015; Peixoto et al. 2017; National Academies of Sciences, Engineering, and Medicine & Ocean Studies Board 2019; Gouezo et al. 2019). The potential use of coral probiotics is one option (National Academies of Sciences, Engineering, and Medicine & Ocean Studies Board 2019), which involves manipulating corals' association with its resident microorganisms to provide benefits to the host, prevent stress, or facilitate recovery (Peixoto et al. 2017). This method assumes that microbiome changes will assist in the acclimation or adaptation of the holobiont to new environmental conditions through the active manipulation of those populations (Coral Probiotic Hypothesis; Reshef et al. 2006). However, there is limited understanding as to whether Symbiodiniaceae communities in adult corals are amenable to change using probiotics, or if their addition supports coral resilience through increased host fitness.

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A diversity of Symbiodiniaceae taxa may co-occur within the coral host simultaneously, ranging from dominant (high) to background (low) abundances (Fabina et al. 2012; Ladner et al. 2012; Putnam et al. 2012; Silverstein et al. 2012; Boulotte et al. 2016). There is evidence that adults of some coral species can alter their symbiont communities to quickly acclimate to changing environmental conditions (Buddemeier & Fautin 1993; Boulotte et al. 2016; Torda et al. 2017). This process involves either uptake of novel exogenous Symbiodiniaceae from the environmental pool ("switching"), or a change in relative abundances of Symbiodiniaceae already present within the coral ("shuffling") (Baker 2003) which appears to be a more common process (Jones et al. 2008; Cunning & Baker 2013; Bay et al. 2016), whereas the evidence for switching during heat stress is limited (Boulotte et al. 2016), and may not be maintained long term (Coffroth et al. 2010). The ability to shuffle or switch may be limited by host genetic constraints and/or the environmental scarcity of certain symbiont taxa (Poland & Coffroth 2017; Quigley et al. 2017*a*, 2017*b*).

Algae in the family Symbiodiniaceae are taxonomically and physiologically diverse, with thermal tolerances ranging between 20°C and 33°C (Silverstein et al. 2015; Grégoire et al. 2017; Swain et al. 2017). *Durusdinium* (formerly clade D) is generally classified as thermally tolerant, whereas *Cladocopium* (formerly clade C) is considered more sensitive (Baker 2003; Berkelmans & van Oppen 2006; Quigley et al. 2018*b*; but see Hume et al. 2015 for *Chaetomium thermophilum*). Shuffling to *Durusdinium* dominance for example may provide increased heat tolerance and allow corals to rapidly increase their acclimation and/or adaptive potential (Fay & Weber 2012). However, some coral species may not be capable of naturally acclimatizing or adapting fast enough to keep pace with the rate of climate warming (Csaszar et al. 2010). Hence, new interventions are promoted to assist corals' natural recovery potential by manipulating the symbiont community to one more amenable to increasing ocean temperatures (bacterial or Symbiodiniaceae) (van Oppen et al. 2015; Torda et al. 2017). It is currently unknown if corals can acquire exogenous symbionts, sustain these changes, or if these changes mitigate bleaching stress and mortality. To address this question, we experimentally exposed the common branching coral *Acropora millepora* to a simulated bleaching event equivalent to 2.43°-heating-weeks (DHW), while supplementing coral fragments with cultured *Durusdinium trenchii* or *Cladocopium goreaui* cells.

Methods

As host-symbiont specificity is a genetically determined trait (Quigley et al. 2017*b*), a single *Acropora millepora* colony was used to control for genotypic effects in Symbiodiniaceae acquisition and regulation. The colony was collected from Davies Reef (−18°49′30″S, 147°38′42″E) on the central Great Barrier Reef (GBR) in 2016 (Permit Number: G12:35236.1) and kept at Central GBR conditions (27.5°C) in the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (AIMS). After 16 months of acclimation in the SeaSim, the colony was fragmented into individual approximately 8 cm fragments (hereafter referred to as "nubbins") and allowed to recover for 4 weeks before the beginning of the experiment, which ran for a total of 20 days (day 1 beginning when the temperature reached 32°C). Temperature ramping increased from 27.5°C to 32°C over 9 days (0.5°C/day) for all tanks excluding the ambient tanks at 27.5°C. The temperature was sustained at 32°C for a further 5 days before being brought back down to 27.5°C (1°C/day). A recovery period began when the temperature had been brought back down to 27.5°C and was maintained for 11 days to monitor recovery and post-thermal stress. Water inflow to tanks was 0.8 L/second and circulated via an internal pump. The light for the aquariums were on a 24 hour 12:12 light/dark cycle, 2-hour sunrise/sunset ramp time, photosynthetically active radiation (PAR) = 100. There were three replicate tanks for each of the four treatments (nine replicate tanks, 12 tanks in total) with three nubbins in each tank.

Durusdinium trenchii (SCF082) and *Cladocopium goreaui* (SCF055-01.10) were cultured and grown in single-host inoculums in stock culture at 27°C at the Algal Culturing Facility at AIMS (see Supplement S1). These taxa were chosen as they represent a known thermally tolerant taxa (*D. trenchii*) and a less thermally tolerant taxa (*C. goreaui*). *D. trenchii* and *C. goreaui* are commonly found in *A. millepora* in central, offshore GBR environments (Ulstrup & van Oppen 2003; Abrego et al. 2009). *A. millepora* nubbins were subjected to heat stress to induce an ecologically relevant bleaching response, equivalent to 2.43 DHW (United States National Oceanic and Atmospheric Administration). The experimental treatments were as follows: (1) elevated temperature + 4.5°C = 32°C and addition of *D. trenchii* (abbreviated as temperature and probiotic treatment: 32/+D1), (2) elevated temperature and addition of *C. goreaui* (32/+C1), (3), elevated temperature but no added Symbiodiniaceae (32/−), and (4) no temperature elevation but with the addition of *D. trenchii* (27.5/+D1).

Coral nubbins were inoculated with Symbiodiniaceae (1.22×10^8 cells/mL) once every 3 days after ramping to 32°C was complete, for a total of four inoculations following established methods (Coffroth et al. 2010). During Symbiodiniaceae inoculation, each tank was drained to 17 L (1 cm above coral nubbins), water was turned off, and 160 mL of Symbiodiniaceae culture were dosed to each respective treatment tank. Each inoculation consisted of Symbiodiniaceae suspended in filtered seawater (0.5 µm) with nutrient-rich IMK media (Supplement S1 & S5). The negative control tanks (32/−) were also dosed with the same 160 mL of filtered seawater and IMK concentration but excluded Symbiodiniaceae. The water was left at 17 L for 2 hours to allow for the potential uptake of the Symbiodiniaceae. This duration was chosen to minimize the stress to corals due to low water turnover but allow for potential uptake. During this time, the tanks were thoroughly stirred to ensure the Symbiodiniaceae cells were mixed. Tanks were then visually inspected to make sure cells were not fixed to the sides or bottom of the tanks. The cells remained in the tanks when the pumps and water flow were turned back on. Cell densities were calculated using a Neubauer hemocytometer (Supplement S1).

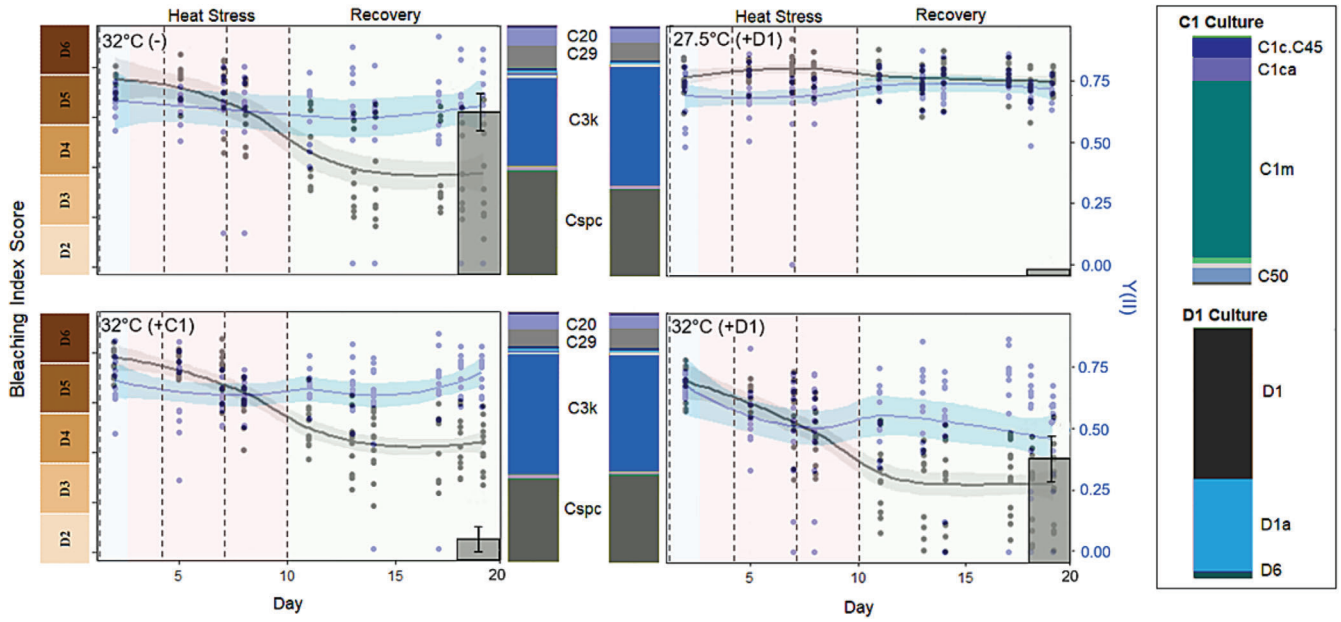

Figure 1. *Acropora millepora* physical responses to experimental bleaching and Symbiodiniaceae probiotic treatment. Bleaching index score and effective quantum yield (YII) are represented in gray and blue, respectively. Gray bars represent percent mortality (±SE) of nubbins measured on the final day. Dashed lines represent Symbiodiniaceae inoculations. Prior to heat stress, during heat stress, and the recovery period are represented in blue, red, and green shading, respectively. Bar plots represent the relative abundances of ITS2 amplicon sequencing of coral nubbins and cultured Symbiodiniaceae cells.

Photographs were taken of each nubbin (at the same time every second day; between 11:00–12:00 hours) to assess bleaching and health status using the Coral-Watch Health Monitoring Chart (Siebeck et al. 2006). A bleaching index score (BIS) (0 = bleached and 6 = healthy) was calculated based on images analyzed in ImageJ (Schneider et al. 2012) and color calibration curves (see Quigley et al. 2019 for details). Pulse amplitude modulated fluorometry was used to determine the effective quantum yield (Y(II) of photosystem II within Symbiodiniaceae cells; Warner et al. 2010; Schrameyer et al. 2016) (see Supplement S2 for more information). Mortality was quantified daily. All statistics were completed in R (version 3.4.1; R Core Team, 2017) (see Supplement S3 for full statistical information).

At the end of the experiment, each coral nubbin was frozen and kept at −80°C. Samples were collected by scraping approximately 30 mg of tissue from each nubbin. DNA was extracted from nubbins and *D. trenchii*- and *C. goreaui*-cultured cells using a sodium dodecyl sulfate method (Wilson et al. 2002). The DNA was then kept at −40°C. Library preparation and sequencing of the internal transcribed spacer (ITS-2) region was performed as described in Quigley et al. (2019). Index polymerase chain reaction amplified products were sequenced at the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia) using Illumina MiSeq 300 bp paired-end sequencing. Bioinformatic processing was performed using the DADA2 and Phyloseq pipeline modified for Symbiodiniaceae (McMurdie & Holmes 2013; Callahan et al. 2016; Quigley et al. 2019) (see Supplement S4).

Results

Acropora millepora nubbins in the 32°C treatment with no added Symbiodiniaceae (32/−) displayed the highest mortality (mean mortality ± SE at 20 days: 66.7 ± 8%), lowest effective quantum yield relative to initial values (mean Y(II) ± SE at 20 days: 0.64 ± 0.03), and substantial bleaching (mean BIS ± SE at 20 days: 3.8 ± 0.31) by the end of the experiment (Fig. 1). These results demonstrate that 2.43 DHW are sufficient to produce substantial experimental heat stress in *A. millepora* nubbins. In contrast, nubbins in the 27.5°C treatment provisioned with *Durusdinium trenchii* (27.5/+D1) exhibited no mortality (0%), high and stable Y(II) values (0.72 ± 0.01), and little bleaching (BIS: 5.7 ± 0.05). The lack of bleaching signs and mortality in the 27.5/+D1 treatment suggests that exposure to *D. trenchii* without any heat exposure does not adversely affect *A. millepora* physiology.

Coral nubbins in the 32°C treatment provisioned with *Cladocopium goreaui* (32/+C1) exhibited the lowest mortality (9.1 ± 5%) and bleaching (BIS: 4.12 ± 0.15) compared to any of the other heat treatments by the end of the experiment (generalized linear mixed model [GLMM], 32/+D1: $p < 0.0001$; 32/−: $p = 0.0023$) (Fig. 1). It was also the only 32°C treatment which displayed signs of recovery of Y(II) from day one (0.69 ± 0.03) to day 20 (0.71 ± 0.02). *A. millepora* nubbins in the 32°C treatment with added *D. trenchii* (32/+D1) exhibited substantial mortality (41.7 ± 9%), had significantly lower Y(II) (0.43 ± 0.04) compared to any other treatment (GLMM, 27.5/+D1: $p < 0.0001$; 32/−: $p = 0.0047$; 32/+C1: $p < 0.0001$)

and experienced the most severe bleaching (BIS: 3.37 ± 0.23) by the end of the experiment (Fig. 1).

The Symbiodiniaceae communities sequenced from the coral nubbins and cultured cells were distinct, suggesting that symbiosis establishment did not occur (Fig. 1 & S1). Although Cspc and C3j variants were shared between the *C. goreaui* culture and all the nubbins, these variants were already present within the corals as indicated by the 32/− treatment (Fig. S1). The coral nubbins were typified by 14 amplicon sequence variants (ASVs or, hereafter, variants) from *Cladocopium*, particularly dominated by C3k and Cspc. No *Durusdinium* variants were detected in any coral tissue samples. Nine variants were found within the *C. goreaui* culture and dominated by ASVs from C1m, C1ca, C1c.C45, and C50. Five *Durusdinium* variants dominated the *D. trenchii* culture.

Discussion

Enhanced bleaching tolerance has been associated with *Cladocopium goreaui* relative to *Durusdinium trenchii* in coral juveniles of some Indo-Pacific acroporid species (Abrego et al. 2008). *C. goreaui* may be able to outcompete *D. trenchii* in hospite through enhanced nitrogen acquisition (Baker et al. 2013) or through increased carbon sharing compared to *D. trenchii* (Cantin et al. 2009), which can confer an energetic advantage to the host, leading to significantly lower mortality in this treatment. Although we did not detect any novel *C. goreaui* sequences within our treatments, the transient usage of these cells may have provided *Acropora millepora* nubbins with some benefit reflected in the decreased mortality and increased photosynthetic efficiency (Connolly et al. 2012). Once warming subsides, thermally tolerant symbiont associations generally do not persist (Thornhill et al. 2006; LaJeunesse et al. 2009; Baker et al. 2013; Kemp et al. 2014) and may explain why novel *C. goreaui* and *D. trenchii* sequences were not retrieved at the end of the experiment.

Symbiodiniaceae dynamics and propensity for shuffling have been shown to be dependent on the severity of thermal stress, initial symbiont community composition, and recovery temperature (Cunning et al. 2015). For example, Coffroth et al. (2010) reported the successful uptake of novel Symbiodiniaceae after experimental bleaching of *Porites divaricata*, although this symbiont community was not stable during recovery. The successful uptake of novel symbionts in this species may be due to the extreme level of bleaching experienced by *P. divaricata* nubbins (loss of 98–99% of symbiont cells) (Coffroth et al. 2010). The *A. millepora* nubbins in this current study did not reach this level of bleaching (BIS > D3 compared to BIS = D1) and therefore higher bleaching severity (> 2.43 DHW) may be needed to induce uptake of novel Symbiodiniaceae in adult *A. millepora*. The relatively low DHW (2.43) may also have been too mild a stressor to induce a fitness advantage in switching or shuffling to D1 (Cunning et al. 2015) given threshold temperatures for this change are likely higher (4 DHW) (Bay et al. 2016). Therefore, shuffling conditions (i.e. sustaining at 32°C for longer period or a warmer recovery period) may be necessary to induce a fitness advantage and therefore lead to probiotic efficacy. Inoculations may also have occurred too late in the experiment to assist in bleaching resilience given heat can trigger a shift in relative abundance prior to bleaching (Thornhill et al. 2006; LaJeunesse et al. 2009; Coffroth et al. 2010; Kennedy et al. 2015). Hence, it may be more beneficial to apply probiotics prior to the increase in temperature (Peixoto et al. 2017). Future studies should assess amplicon sequencing of the relative abundances of Symbiodiniaceae prior to heat stress, during stress, and during recovery to gain further understanding of symbiont community flexibility and potential benefits of these probiotics.

The provisioning of *C. goreaui* cells significantly reduced bleaching-related mortality compared to controls, although reduced mortality and improved photosynthetic efficiency may have been derived from nutritional supplementation and not symbiosis reestablishment. While associations with thermally tolerant symbionts have been found to be beneficial to the coral host (Fay & Weber 2012; Boulotte et al. 2016), the establishment of these Symbiodiniaceae taxa did not occur. During a bleaching event, corals can rely on heterotrophic feeding and alternative energy sources to survive (Grottoli et al. 2006; Bessell-Browne et al. 2014). For example, decreased bleaching susceptibility and increased pigmentation during recovery was found in experimentally heated and fed corals when compared to non-fed corals (Ferrier-Pagès et al. 2010; Connolly et al. 2012). Heterotrophic feeding was likely absent in the 32°C treatment with no added Symbiodiniaceae cells as the seawater was filtered to 0.5 μm (therefore excluding any free-living Symbiodiniaceae and other microbial food sources) (LaJeunesse 2002), potentially explaining the high levels of bleaching.

The ability of scleractinian corals to use dinoflagellates as a food source has not been thoroughly investigated as most studies have addressed corals' ability to ingest zooplankton algae (Sorokin 1973; Leal et al. 2014), although *Symbiodinium microadriaticum* has been used for clam nutrition (Fitt et al. 1986). Digestion of Symbiodiniaceae from within the polyp tissues has been reported, but it is unclear whether this may support colony survival under stressful conditions (Titlyanov & Titlyanova 2002). The 32°C treatment provisioned with *C. goreaui* exhibited significantly lower mortality compared to corals provisioned with *D. trenchii*, suggesting that if feeding of the Symbiodiniaceae cells was occurring, then there may be a difference in nutritional benefit between these taxa. Different Symbiodiniaceae taxa have been found to harbor varying lipid bodies (Tchernov et al. 2004) and metabolite profiles (Klueter et al. 2015), which may confer different nutritional benefits for potential tissue growth and metabolism (Hawkins & Klumpp 1995). Preferential feeding on Symbiodiniaceae from the coral host has not yet been investigated; hence, uncertainty remains whether nutritional supplementation was responsible for the differences in mortality across treatments.

Recent heritability estimates suggest substantial host genetic regulation of the Symbiodiniaceae community during early coral life-history stages, which may extend into adulthood (Quigley et al. 2017*b*, 2018*b*; Poland & Coffroth 2019). High heritability of bleaching, mortality, and Symbiodiniaceae community diversity (Kenkel et al. 2015) and the strong influence

of host identity (i.e. genet) (Kenkel et al. 2013, 2015; Cunning et al. 2015; Drury et al. 2017) also suggest substantial host limitation to acquiring and maintaining novel Symbiodiniaceae communities, even in horizontally transmitting coral species. To control for these known genotypic effects in uptake, only one colony (genet) was used. It will be important in subsequent work to include multiple *A. millepora* genotypes to each treatment so that any differences between treatments and variation among genotypes may be tested to assess the generality of these conclusions at the population and species level.

Finally, Symbiodiniaceae are also readily available in the environmental pool, whereas in this study, corals were selectively inoculated at certain times for short periods (2 hours). Therefore, this may not have allowed sufficient time for uptake. Direct effects due to the provisioning of distinct Symbiodiniaceae cannot be excluded and may explain the observed variability in survival with the addition of different symbiont taxa. The provisioning of *C. goreaui* may have also had secondary effects on other members of the microbial consortia, i.e. the increased abundance of bacterial partners associated with *C. goreaui* (Bernasconi et al. 2019), that then indirectly influence host recovery (Ziegler et al. 2017). A future challenge will be to uncover how Symbiodiniaceae probiotics may influence and potentially benefit corals at the cellular level under both ambient and stressful conditions.

The results presented demonstrate that the application of Symbiodiniaceae probiotics significantly reduces bleaching related mortality when corals are provided either *D. trenchii* or *C. goreaui*. *A. millepora* inoculated with *C. goreaui* exhibited lower mortality, less bleaching, and higher photosynthetic efficiency in comparison to corals inoculated with *D. trenchii*. Interestingly, the exogenous uptake of symbionts was not observed, and future work is needed to assess the potential nutritional benefit of Symbiodiniaceae probiotics as well as the host regulation which controls symbiosis establishment. As coral reefs continue to decline, the continued assessment and development of restoration interventions will be critically important in this period of rapid environmental change.

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Supporting Information

The following information may be found in the online version of this article:

Supplement S1. Experimental design and inoculation
Supplement S2. Data collection (Bleaching Index Scores and PAM).
Supplement S3. Statistical analysis.
Supplement S4. DNA extraction and analysis.
Supplement S5. IMK stock standard composition.
Figure S1. Relative abundance of Symbiodiniaceae sequence variants by treatment.

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