Changes in microbial communities, photosynthesis and calcification of the coral *Acropora gemmifera* in response to ocean acidification

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With the increasing anthropogenic CO₂ concentration, ocean acidification (OA) can have dramatic effects on coral reefs. However, the effects of OA on coral physiology and the associated microbes remain largely unknown. In the present study, reef-building coral *Acropora gemmifera* collected from a reef flat with highly fluctuating environmental condition in the South China Sea were exposed to three levels of partial pressure of carbon dioxide (pCO₂) (i.e., 421, 923, and 2070 μatm) for four weeks. The microbial community structures associated with *A. gemmifera* under these treatments were analyzed using 16S rRNA gene barcode sequencing. The results revealed that the microbial community associated with *A. gemmifera* was highly diverse at the genus level and dominated by Alphaproteobacteria. More importantly, the microbial community structure remained rather stable under different pCO₂ treatments. Photosynthesis and calcification in *A. gemmifera*, as indicated by enrichment of δ¹⁸O and increased depletion of δ¹³C in the coral skeleton, were significantly impaired only at the high pCO₂ (2070 μatm). These results suggest that *A. gemmifera* can maintain a high degree of stable microbial communities despite of significant physiological changes in response to extremely high pCO₂.

Rising CO₂ in the atmosphere elevates the partial pressure of carbon dioxide (pCO₂) in seawater and reduces the global oceanic pH and carbonate ion concentrations, which is called ocean acidification (OA). It has been suggested that OA has profound effects on marine organisms and ecosystems, particularly calcifying organisms such as reef-building corals¹-³. With the increasing OA associated with ocean warming due to the rising global CO₂ emissions², there is an urgent need to understand and predict the tolerance and response of corals to future climate change.

Reef-building corals are commonly referred to as holobionts, which comprise coral host and associated microorganisms including endosymbiotic photosynthetic algae, bacteria, and archaea, among others. These complex microbial partners play pivotal roles in coral health and holobiont function in carbon, nitrogen and sulfur cycles. The future fate of coral reefs largely depends on the capacity of corals and their symbionts to acclimatize or adapt to climate change.²⁴-⁷ There is emerging evidence that corals can adapt to climate change⁴, although coral photosynthesis and growth can be negatively impacted by OA⁵. Moreover, flexible coral-algal symbiosis may facilitate the acclimatization/adaptation of the holobiont through algal shuffling or switching⁶¹¹.

Shifting in the composition of coral-associated microbiota has been observed following environmental disturbances (e.g., elevated temperature) and has often been linked to impaired host health¹²-¹³, although it has also been hypothesized to mediate holobiont resistance to environmental perturbations⁶¹-¹². Coral-associated microbial communities may be affected directly or indirectly by OA and would subsequently compromise holobiont fitness (i.e., changes in photosynthesis or calcification) and survival, possibly due to a shift in the functional roles of microbial associations¹²,¹⁶-¹⁸. However, to date it is not clear how microbial communities change in coral in

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response to OA, or whether these changes also alter host physiology. Preliminary laboratory-based investigations have revealed a remarkable impact of increased pCO2 or reduced pH on coral microbial communities12,16,18. In contrast, no significant changes were observed in the microbial communities of transplanted corals in natural CO2 vents19 and associated with two Pacific corals after 8 weeks of exposure to increased pCO220. These contradictory findings underscore the need for further research. Most recently, microbial communities associated with coral and sponge from natural CO2 seeps have demonstrated species-specific acclimatization to their habitats21. Therefore, the potential of natural microbial communities in corals to acclimatize/adapt to OA cannot be overlooked.

Natural fluctuations in seawater pH/pCO2 are common, especially diel pH/pCO2 fluctuations in shallow water coral reefs22 and these fluctuations affect the abundance and distribution of marine organisms23. The fauna have been suggested to be locally acclimatized/adapted to the variable pH environment as an evolutionary mechanism to cope with future acidification24–26. However, the adaptation of the coral holobionts to OA remains largely unexplored and is worth careful investigation.

The Luhuitou fringing reef (18°12′N, 109°28′E) is located in the southern Hainan Island, South China Sea (see Supplementary Fig. S1) and used to have a high coverage of living coral, which has declined by 80% since the 1960s27. The diurnal and seasonal variation of the reef flat seawater pH/pCO2 is high28–30, and the recorded extreme level is even lower than the value currently predicted at the end of this century (see Supplementary Table S1). The rapidly-growing branching coral Acropora, which is distributed worldwide, is an ecologically important genus in this reef flat. In the present study, A. gemmifera colonies collected from this reef flat were exposed to three pCO2 levels to test our hypothesis that both the coral physiology and the microbial communities associated with this coral species are stable and resistant to OA exposure.

Results and Discussion

Overview of the microbial communities. After quality filtering, 308,591 reads were used for the downstream analyses. The number of operational taxonomic units (OTUs), Chao1 estimation of species richness, and Shannon index were obtained at a dissimilarity of 3% (Table 1). The rarefaction analyses revealed that the sequencing effort for each sample was sufficient to reflect the microbial diversity, and the rank-abundance curve showed that most OTUs had an abundance lower than 0.1%, which demonstrated that the microbial communities were occupied by rare species (see Supplementary Fig. S2). The prevalence of rare species has been widely demonstrated elsewhere, yet the ecological and functional roles of these rare species remain unknown31. There were no significant differences in beta diversity among the pCO2 treatments, which is in contrast to the findings of a previous report showing an increase in coral microbial diversity with decreasing pH, possibly caused by an intermediate disturbance32.

In total, 24 bacterial and 2 archaeal phyla were detected in the coral and seawater samples, including Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Thaumarcheota and Euryarchaeota (see Supplementary Fig. S3). The relative abundance of archaea made up less than 0.1% of the seawater samples, with most OTUs belonging to autotrophic ammonia-oxidizing archaea (AOA) within the phylum Thaumarcheota, which was even less abundant in the coral samples. It has been suggested that archaea may play prominent roles in corals and reefs3, although their abundance in both corals and reef water is much lower than that of bacteria33. Notably, the bacterial communities in both A. gemmifera and seawater were dominated by Proteobacteria and the most abundant class was Alphaproteobacteria (56–80%), among which the majority were assigned to the family Brucellaceae in the order Rhizobiales (Fig. 1) followed by Gammaproteobacteria (9–26%). Both Alphaproteobacteria and Gammaproteobacteria are commonly highly abundant in corals, but their relative abundance varies among species33.

Table 1. Sample information and summary of microbial communities in corals and seawaters. “H”, “M” and “C” refer to high, medium and control pCO2 treatment, respectively. “A” and “W” refer to coral and seawater samples, respectively. The number following the letter indicates a replicate. Chao1, Observed species and Shannon index were determined at 3% dissimilarity after normalizing the full 16S dataset (including bacterial and archaeal sequences) to 12,459 sequences per sample.

| Sample ID | Treatment            | Number of qualified reads | Total OTUs    | Chao1 Ave. | Observed species Ave. | Shannon Ave. |
|-----------|----------------------|---------------------------|---------------|------------|-----------------------|--------------|
| HA.1      | High pCO2 treatment  | 37600                     | 1301          | 1678.248   | 707.2                 | 5.420        |
| HA.2      |                      | 19098                     | 1115          | 1826.916   | 874.8                 | 5.494        |
| HA.3      |                      | 29468                     | 1687          | 2102.160   | 1087                  | 6.333        |
| MA.1      | Medium pCO2 treatment| 27489                     | 1108          | 1545.223   | 707.2                 | 5.360        |
| MA.2      |                      | 27946                     | 1295          | 1780.889   | 822.9                 | 5.316        |
| MA.3      |                      | 26650                     | 1074          | 1528.017   | 709.3                 | 5.431        |
| CA.1      | Control pCO2 treatment| 24616                 | 1665          | 1629.961   | 716.8                 | 5.258        |
| CA.2      |                      | 21224                     | 989           | 1576.258   | 727.8                 | 5.527        |
| CA.3      |                      | 28163                     | 1244          | 1688.842   | 783.5                 | 5.450        |
| HW.1      | High pCO2 treatment  | 14358                     | 1237          | 1901.201   | 1152.6                | 5.252        |
| MW.1      | Medium pCO2 treatment| 15688                     | 1203          | 1795.261   | 1075.2                | 4.860        |
| CW.1      | Control pCO2 treatment| 12463                   | 1127          | 1886.261   | 1126.7                | 5.908        |
Taxonomic assignment at the genus level was summarized, and genera with an abundance of greater than 1% in at least one sample are shown in a heat map (Fig. 2). In the present study, the unclassified Brucellaceae (>24%), Acinetobacter (>9%) and Pannonibacter (>5%) were the most abundant genera in coral, regardless of the pCO2 treatment. Diazotrophs within the order Rhizobiales have been found in other coral species and were considered to be important for coral holobiont in nitrogen-limited waters. It has been shown that copiotrophic taxa including Brucellaceae were enriched in algal-dominated environment. Diverse algal communities on the Luhuitou fringing reef might contribute to the dominance of unclassified Brucellaceae in A. gemmifera. Acinetobacter spp. have also been commonly reported in bleached and healthy corals. Therefore, it is reasonable to suggest that the dominant genera, including Acinetobacter and the unclassified Brucellaceae, play critical roles in A. gemmifera. Interestingly, the putatively endosymbiotic Endozoicomonas was detected at a very low level in all coral samples (<2%). The photosynthetic Cyanobacteria assigned to the genus Synechococcus have been reported in sponge and coral and were also detected at a very low abundance (0.2%) in A. gemmifera. However, the functions of bacteria and archaea and their interactions in the coral holobiont remain largely unclear.

Stability of microbial communities in A. gemmifera. As estimated by Adonis analysis at all taxonomic levels (Adonis test, p > 0.05) and nMDS ordination (see Supplementary Fig. S4), there were no significant differences in microbial community compositions in A. gemmifera among the different pCO2 treatments even after a 4-week exposure. Additionally, results from the SIMPER analysis showed that the dissimilarity of microbial communities among pCO2 treatments was very small (see Supplementary Fig. S5). Taken together, these findings suggest that the A. gemmifera microbiome was not significantly affected by elevated pCO2 and could remain relatively stable (Fig. 2). This result is inconsistent with the findings of some previous studies in which the coral microbiome shifted under higher pCO2 or lower pH treatments over treatment periods ranging from days to months. However, our finding is consistent with some other studies. For example, there were no differences in the microbial community structure in coral between pH 7.7 (pCO2 = 1187 μatm) and 7.5 (pCO2 = 1638 μatm) whereas a significant difference was observed between pH 8.1 (pCO2 = 464 μatm) and 7.9 (pCO2 = 822 μatm).
after 6 weeks of CO₂ exposure. Moreover, Meron et al. observed no significant changes in microbial communities associated with two Mediterranean coral species that were transplanted along natural pH gradients. A recent study reported that the microbial communities of two Pacific coral species were tolerant to reduced pH 7.9 (pCO₂ 738–835 μatm). These inconsistent results might reflect that some coral-microbial associations are more resistant to increases in pCO₂/decreases in pH than others, but these findings could also be partially attributed to differences in the experimental conditions (e.g., field vs laboratory, pCO₂ or pH level, among others), and the exposure duration.

In most cases, microbial communities are dynamic and can rapidly respond to OA in seawater, biofilms, and other associated systems. The genus Acropora is among the most sensitive coral to environmental change. The potential for coral acclimatization or adaptation to climate change has been studied, and the physiological and molecular mechanisms responsible for OA resistance have recently been proposed. Although there is limited evidence for biological adaptation to climate change in coral microbial symbionts, the adaptive power to climate change has been well documented in the photosymbiotic Symbiodinium. The shallow habitat of the coral Acropora gemmifera sampled in the present study has been experiencing regular large diurnal and seasonal variations in pH/pCO₂ (see Supplementary Table S1), which are mainly driven by biological activities of the reef. Therefore, it is most likely that microbial communities harbored by the natural population of A. gemmifera are resistant to the increased pCO₂, due to long-term acclimatization/adaptation to the highly dynamic pH conditions within the reef flat. Thus, there may be a resilient relationship between coral and microbial partners that can help corals overcome the fluctuations in seawater pH/pCO₂. However, we note that the stability of the coral microbiome is based on only one species collected from a fluctuating environment. The application of variable pCO₂ conditions and controls from stable pH/pCO₂ environments in highly replicated culture experiments with consideration of tank effects could further confirm this assumption in future studies.

A recent study supports this interpretation. Morrow et al. found that microbial communities associated with coral and sponge originally from natural volcanic CO₂ seeps were distinct from the nearby control sites, reflecting the acclimatization of the host-symbiont to the high pCO₂ environment. Local acclimatization/adaptation to environmental variations in pCO₂, temperature and nutrients, among others, has revealed the capabilities of marine organisms including reef-building corals and symbiotic algae, to adapt to future climate change.
Increased (923 μatm and 2070 μatm) pCO₂ conditions. Skeletal isotopic composition of non-photosynthetic coral Tubastrea sp. and photosynthetic coral Pavona sp. reported in a previous study (McConnaughey 1989) are plotted for comparison. Photosynthesis indicated the carbon isotopic offset due to respiration and photosynthesis. “Slow” and “Fast” indicated slow and fast coral calcification rates. Fast growing Acropora corals are expected to have more enriched δ¹³C but depleted δ¹⁸O values.

However, in general, the species-specific response of marine organisms to OA remains poorly understood. Thus, it is rather premature to conclude whether we can extrapolate the adaptive power of coral and its associated microbes documented in the present study to other coral species living in highly fluctuating reef environments.

Skeletal isotopic response to ocean acidification. During our experiments, all blocks of A. gemmifera exposed to the different pCO₂ treatments grew, survived and formed new skeleton (see Supplementary Fig. S6), even at the high pCO₂ (pH reduced to 7.47). When the fast-growing coral A. gemmifera skeletal δ¹³C and δ¹⁸O were compared among the different pCO₂ treatments, the skeletal δ¹³C values in A. gemmifera were significantly different between any two pCO₂ treatments except between the control and the medium (Fig. 3). Skeletal δ¹³C values were depleted by 1.10‰ and 1.04‰ for the control vs. high pCO₂ and for the medium vs. high pCO₂, respectively (one-way ANOVA, Tukey test, p < 0.05). Skeletal δ¹⁸O values in A. gemmifera were enriched with increased pCO₂: they were 0.55‰ and 0.38‰ heavier in response to high pCO₂ than those in the control and medium, respectively (one-way ANOVA, Tukey test, p < 0.05). Compared with the previous data, skeletal δ¹⁸O values revealed greater depletion in fast-growing coral A. gemmifera, while the skeletal δ¹³C values remained within range. In addition, the relationship between skeletal δ¹³C and δ¹⁸O in the non-photosynthetic coral Tubastrea sp. deviated the most from those both in the photosynthetic corals A. gemmifera and Pavona sp. (Fig. 3).

The isotopic composition of the coral skeleton can be affected by metabolic isotope effects (e.g., photosynthesis and respiration) and kinetic isotope effects (e.g., the calcification process). The coral skeletal δ¹³C and δ¹⁸O were generally used as an effective proxy to study photosynthesis, respiration and calcification processes. In general, photosynthesis and calcification can enrich skeletal δ¹³C but deplete skeletal δ¹⁸O due to isotope fractionation. Compared non-photosynthetic (Tubastrea sp.) and photosynthetic (Pavona sp.) corals, the relationship of δ¹³C vs. δ¹⁸O in Pavona sp. and A. gemmifera was different from that in Tubastrea sp. (non-photosynthetic coral) due to active photosynthesis. In addition, more δ¹⁸O deviation was observed in A. gemmifera than Tubastrea sp. and Pavona sp., mostly due to the highest growth rate in A. gemmifera (Fig. 3). Skeletal δ¹³C values in A. gemmifera were significantly depleted at the high pCO₂, suggesting that the photosynthetic rates were much lower at the high pCO₂ than at the control pCO₂. The variation in skeletal δ¹⁸O values of A. gemmifera was consistent with the findings of a previous study demonstrating an enrichment of δ¹⁸O in the coral skeleton in response to elevated pCO₂. The coral calcification rate decreases under reduced pH conditions, which corresponds to heavier skeletal δ¹⁸O whereas low pCO₂ and higher pH lead to species with lighter δ¹⁸O because HCO₃⁻ is isotopically heavier than CO₃²⁻. Consequently, the significantly enriched δ¹⁸O and more depleted δ¹³C in A. gemmifera observed herein may reflect slight reductions in photosynthesis and calcification at the high pCO₂. It should be noted that A. gemmifera skeletal δ¹³C and δ¹⁸O values did not vary significantly at the medium pCO₂, potentially because this stress level did not exceed its acclimatization range. These findings indicate that the coral A. gemmifera is able to acclimate to an acidifying ocean, even in the presence of a dramatically increasing atmospheric CO₂ concentration.

Although the mechanisms by which extremely high pCO₂ induces decreased photosynthesis and calcification efficiencies in A. gemmifera are unknown, several potential mechanisms have been proposed, such as photoinhibition and suppression of the carbon concentrating process. Photosynthesis, calcification and other physiological processes in reef-building corals can be influenced by their microbial partners or vice versa under OA. However, the microbial communities associated with A. gemmifera remained unchanged as a consequence.
of host physiological changes, further supporting our hypothesis that highly stable microbial associations are likely driven by local acclimatization/adaptation to the fluctuating environment. Alternatively, host physiological costs might result from a potentially increasing energy demand to maintain stable microbial assemblages at the extremely high pCO₂ that exceeds its tolerance level.

It has also been proposed that physiological differences among symbiotic algal phylotypes may influence the stable isotopic composition of coral skeleton. Furthermore, the distinct mechanisms used to concentrate carbon by different Symbiodinium phylotypes and their physiological responses to OA are phylotype-specific. For example, Symbiodinium community shifts may occur in response to environmental stresses. In the present study, we did not investigate Symbiodinium phylotypes associated with A. gemmifera. A recent study found no changes in Symbiodinium phylotypes associated with corals among different pH conditions, suggesting the presence of stable Symbiodinium assemblages in corals in response to OA. In general, a stable microbial partnership to maintain key metabolic functions can improve coral holobiont acclimatization or adaptation to environmental stress. However, the interactions between microbial communities and coral physiology remain far from clarified.

Conclusions
In this study, the tropical fast-growing coral A. gemmifera from a shallow habitat with natural pH/pCO₂ fluctuations was selected as a representative species and was exposed to a 4-week CO₂ treatment. The microbial communities and skeletal isotopic compositions were examined simultaneously. We found that the microbial communities in A. gemmifera remained remarkably stable. In contrast, neither photosynthesis nor calcification in the coral were impacted under medium pCO₂ but were both negatively affected under extremely high pCO₂, as demonstrated by an enrichment of δ¹³C in the skeleton under extremely high CO₂ stress. The present findings indicate that some reef-building corals may be more tolerant to OA in pH/pCO₂ fluctuating environments and have a high degree of host-symbiont fidelity, despite the observed impairment of host physiological processes in response to high CO₂ stress. This study also contributes to our understanding of the variability of OA resistance among coral-microbial associations. Because coral reefs are facing other environmental stresses in addition to OA, the synergistic effects of multiple stressors on the coral microbiome must be carefully examined to understand the persistence of the coral holobiont and coral reefs in the future ocean.

Methods
Experimental design and sample collection. The experiment was conducted in an outdoor seawater flow-through system at the Tropical Marine Biological Research Station in Hainan (TMBRS) near the Luhuitou fringing reef. Seawater was pumped directly from a depth of 6 m in the front of the TMBRS into three 2000-L header tanks in which the designated pCO₂ level was adjusted with CO₂ gas. Three pCO₂ treatments were projected current pCO₂ levels, those at the end of the present century, and double the end of the present century: 421 μatm, 923 μatm, and 2070 μatm with pH values of 8.07, 7.76 and 7.47, respectively. The well-mixed seawater from the header tank continually flowed into three aquariums at a rate of 0.51 min⁻¹. Each aquarium was equipped with a submerged pump to drive the water flow, and all aquaria were maintained under a natural light–dark cycle to mimic the field condition.

Six healthy colonies of A. gemmifera were collected from the Luhuitou fringing reef at a depth of ~2 m in May 2014 and divided into small pieces. After acclimation for two weeks in large aquaria with running water, one coral nubbin was randomly selected and was suspended using fine nylon strings in three aquaria for exposure to each of the three pCO₂ treatments. The coral nubbins from same colony were evenly distributed among the pCO₂ treatments to avoid any possible sampling bias. A total of 9 coral nubbins were maintained for further analysis during the experiment.

At the end of the experiment (i.e., 4-week exposure), 1 L of seawater from each treatment was filtered through 0.2 μm polycarbonate (PC) membrane filters and stored at −20 °C for further analyses. One coral nubbin from each treatment aquarium was sampled and divided into two aliquots. One was rinsed three times and then preserved in 70% ethanol and stored at −20 °C for DNA extraction; the other was used for stable isotope analyses.

Determination of environmental parameters. Photosynthetically active radiation (PAR) in each aquarium was recorded every 30 min below the seawater surface using the Hobo® logger (Onset, USA). The average diurnal variations in PAR during the 4-week period are shown in Fig. S7. The seawater pH and temperature were measured daily in each aquarium using a pH meter (Orion Star®) and the total alkalinity was also determined weekly using an automated titration system (Metrohm 877 Titrino plus, Switzerland). Carbonate system parameters, including [HCO₃⁻], [CO₃²⁻], pCO₂, and aragonite saturation state (Ωₐ), were calculated from the measured pH and total alkalinity using the CO2SYS program (Table 2).

DNA extraction and 16S rDNA amplicon sequencing. A preserved piece of coral was homogenized in liquid nitrogen and then the total DNA from the resulting coral powder and filtered seawater samples was subsequently extracted using the Fast DNA® SPIN Kit for Soil (MP Biomedicals, Irvine, CA) according to the company’s protocol. The DNA samples were amplified by PCR using barcoded primers targeting the hypervariable region V3-V4 of the 16S rRNA gene of Bacteria and Archaea: 341F (5′- CCTAYGGGRBGCASCAG-3′) and 802R (5′- TACTCGGTTATCTAATCC-3′). The PCR amplification was performed using a thermocycle controller (M Research Inc., Bio-Rad) with the following program: an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. All PCR products were purified using the Qiagen Agarose Gel DNA Purification Kit (Qiagen, Germany) and quantified using a NanoDrop device (Thermo Scientific, USA). All amplicon products were mixed at equal concentrations and
sequenced on an Illumina Miseq platform using 2 × 300 bp mode at Novogene (Beijing, China). The raw reads were submitted to the NCBI Sequence Read Archive under accession number SRP066229 (SRR2917919).

**Sequence data processing.** Overlapping paired-end reads were merged to obtain full-length 16S V3-V4 fragments using PEAR54. After de-multiplexing and quality control, the downstream bioinformatics analysis was performed with QIME1.5.0 pipelines55. Briefly, OTUs with 97% similarity were defined after the qualified reads were clustered using Uclust56. Representative sequences for each OTU were assigned to different taxa using the abundance were summarized at the phylum, class, order, family, and genus levels. The species diversity, Shannon threshold. Representatives assigned to eukaryotes, chloroplasts and mitochondria were filtered out. The taxon and chemistry were calculated using the CO2SYS program.

**Stable isotope analyses.** Skeleton fragments of *A. gemmifera* were soaked in 30% hydrogen peroxide to remove coral tissue and then sonicated for 4 min at 20°C. Skeletons were subsequently washed several times with double-distilled water and dried overnight at 50°C. The newly grown part was scalped and ground into powder. Coral skeletal δ13C and δ18O data were obtained using a Finnigan MAT 253 Isotope Ratio Mass Spectrometer coupled to a Kiel Carbonate Device IV at the South China Sea Institute of Oceanology, Chinese Academy of Sciences, China. δ13C and δ18O were determined by repeated measurements of the international reference standard NBS-18. δ13C and δ18O values were presented as the per mil (%) deviation of 13C/12C and 18O/16O, respectively.

**Statistical analyses.** To test the effect of pCO2 treatments on microbial community compositions of coral samples, after normalizing all sequence reads for taxonomic analysis to the lowest sequencing depth (19,098 reads), pairwise dissimilarities among coral samples were calculated based on the Bray–Curtis index for ‘Adonis’, which is a non-parametric multivariate analysis of variance. In the Adonis analysis, the distance matrix was the response variable with pCO2 treatment as independent variable. Non-metric multidimensional scaling (nMDS) was also performed to visualize the dissimilarities. Genera making the greatest contribution to dissimilarity among the pCO2 treatments were further investigated through similarity percentage (SIMPER) analysis. To compare coral skeletal δ13C and δ18O among pCO2 treatments, one-way ANOVA and Tukey’s test were employed. All analyses were conducted using the package vegan within the R statistical environment (version 3.1.3, R Development Core Team, 2015).

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**Table 2.** Carbonate chemistry parameters of seawater for each treatment. Values are means ± SE. Seawater pH (NBS scale), and salinity (31.5) were measured daily during the experiment (n = 28). Total alkalinity (TA) was measured at a specific time point every week (n = 4). The remaining parameters for carbonate seawater chemistry were calculated using the CO2SYS program.

| Treatment | pH | Alkalinity (µmol kg⁻¹) | HCO₃⁻ (µmol kg⁻¹) | CO₂(µmol kg⁻¹) | pHCO₃ (µatm) | Ora |
|-----------|----|------------------------|-------------------|---------------|-------------|-----|
| Control pCO₂ | 8.07 ± 0.02 | 2233 ± 22 | 1701 ± 35 | 214 ± 15 | 421 ± 49 | 3.6 ± 0.25 |
| Medium pCO₂ | 7.76 ± 0.02 | 2223 ± 11 | 1912 ± 36 | 125 ± 11 | 923 ± 113 | 2.1 ± 0.19 |
| High pCO₂ | 7.47 ± 0.02 | 2230 ± 13 | 2089 ± 25 | 67 ± 6 | 2070 ± 259 | 1.1 ± 0.01 |

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**Carbonate chemistry parameters of seawater for each treatment.** Values are means ± SE. Seawater pH (NBS scale), and salinity (31.5) were measured daily during the experiment (n = 28). Total alkalinity (TA) was measured at a specific time point every week (n = 4). The remaining parameters for carbonate seawater chemistry were calculated using the CO2SYS program.
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**Author Contributions**

G.W.Z. wrote the manuscript with all authors commenting. G.W.Z., T.Y., L.C. and L.J. performed the experiment and undertook carbonate analyses. W.P.Z., R.M.T., H.Y.T. and X.C.Y. participated in data analyses. G.W.Z., S.L., H.H. and P.Y.Q. designed the research.

**Additional Information**

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