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NOTE

Ocean acidification rapidly reduces dinitrogen fixation associated with the hermatypic coral *Seriatopora hystrix*

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ABSTRACT: Since productivity and growth of coral-associated dinoflagellate algae is nitrogen (N)-limited, dinitrogen (N_2) fixation by coral-associated microbes is likely crucial for maintaining the coral–dinoflagellate symbiosis. It is thus essential to understand the effects future climate change will have on N_2 fixation by the coral holobiont. This laboratory study is the first to investigate short-term effects of ocean acidification on N_2 fixation activity associated with the tropical, hermatypic coral *Seriatopora hystrix* using the acetylene reduction assay in combination with calcification measurements. Findings reveal that simulated ocean acidification (pCO_2 1080 µatm) caused a rapid and significant decrease (53%) in N_2 fixation rates associated with *S. hystrix* compared to the present day scenario (pCO_2 486 µatm). In addition, N_2 fixation associated with the coral holobiont showed a positive exponential relationship with its calcification rates. This suggests that even small declines in calcification rates of hermatypic corals under high CO_2 conditions may result in decreased N_2 fixation activity, since these 2 processes may compete for energy in the coral holobiont. Ultimately, an intensified N limitation in combination with a decline in skeletal growth may trigger a negative feedback loop on coral productivity exacerbating the negative long-term effects of ocean acidification.

KEY WORDS: Acetylene reduction assay · Nutrient limitation · Carbon fixation · Calcification · Coral holobiont

INTRODUCTION

Hermatypic corals are highly adapted to the oligotrophic waters in which they occur by forming a mutualistic symbiosis with dinoflagellate algae of the genus *Symbiodinium* (Muscatine & Porter 1977). Although this symbiosis enables an efficient internal recycling of nutrients, new nutrients (particularly bioavailable nitrogen) are needed to sustain net productivity and to compensate the loss of nutrients. New nitrogen (N) is acquired by the coral holobiont via capture of prey, assimilation of inorganic and organic N from the water column, and dinitrogen (N_2) fixation (Lesser et al. 2007, Grover et al. 2008). In this context, Lesser et al. (2004) for the first time detected endosymbiotic cyanobacteria in the coral *Montastraea cavernosa.* Recent research revealed that diazotrophs (N_2 fixing bacteria and archaea) are ubiquitous members of coral-associated microbial communities and form species-specific associations with their hosts (Lema et al. 2012, 2014, Olson & Lesser 2013). N_2 fixation activity has also been detected for several coral

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species, suggesting a high importance of this process in fulfilling the N demand of corals (reviewed in Fiore et al. 2010 and Cardini et al. 2014).

Since growth of *Symbiodinium* spp. is N limited, low dissolved inorganic N (DIN) availability may be essential to maintain the stability of this symbiosis (Falkowski et al. 1993). On the other hand, *Symbiodinium* spp. is efficient in the uptake of fixed N (Kopp et al. 2013), and cell division rates are faster in corals that show high N_2 fixation activity (Lesser et al. 2007). Hence, N_2 fixation may play a key role in regulating the coral–dinoflagellate symbiosis. The effects of environmental changes, such as ocean acidification, on N_2 fixation associated with hermatypic corals have yet to be resolved. Several studies reported reduced calcification rates under high CO_2 conditions and reduced aragonite saturation (Cohen & Holcomb 2009, Ries et al. 2009, Crook et al. 2013). Even though positive as well as negative effects of ocean acidification on N_2 fixation activity by planktonic diazotrophs have been reported (Levitan et al. 2007, Czerny et al. 2009, Shi et al. 2012), there are no studies up to now investigating the effects of ocean acidification on N_2 fixation associated with hermatypic corals. Thus, in the present study we experimentally investigated the short-term response of N_2 fixation and calcification (light/dark) in the exemplary coral holobiont *Seriatopora hystrix* exposed to high CO_2 conditions as they may occur before 2100 according to the Intergovernmental Panel on Climate Change (IPCC) scenario RCP 8.5 (Riahi et al. 2007).

MATERIALS AND METHODS

Model organism and sample preparation

The hermatypic coral *Seriatopora hystrix* was selected as model organism for this study as it is abundant, occurs in a wide range of habitats, and is frequently used in physiological studies (Sheppard 1987, Hoegh-Guldberg & Smith 1989, Bongaerts et al. 2011). The coral used for the experiment was acquired from the company De Jong Marinelife (Netherlands) and was collected from a shallow water depth of about 5 m in Indonesia. One individual colony was fragmented into 30 smaller colonies of an average size of 11.75 ± 1.12 cm^2 (mean ± SE) to remove genetic variability. All fragments were kept in a mesocosm holding tank (2000 l) in the laboratory facilities of the Leibniz Centre for Tropical Marine Ecology (ZMT, Bremen) for 2 mo prior to the measurements.

Experimental incubations

The seawater used for the CO_2 treatment was taken from the coral holding tank, filtered (0.1 µm, AcroPak™) and equilibrated with gas of defined CO_2 concentrations of either 486 ppm by volume (ppmv; ambient) or 1080 ppmv (high). The resulting changes in seawater carbonate chemistry were calculated from pH (NBS) and total alkalinity (TA) using the CO_2 Sys Excel Macro (Lewis & Wallace 1998). pH (NBS) reading was obtained from a multiprobe (WTW 3430) and TA was measured by end-point titration with TW alpha plus (SI Analytics) using 0.5 M HCl. Corals were exposed to the CO_2 treatment in holding tanks for 20 h prior to the first incubations and for 24 h in between the first and the second incubation (salinity 34‰, temperature 26° ± 1°C, PAR 110 ± 5 quanta µmol s^{-1} m^{-2}). Additionally seawater at ambient or high CO_2 was used during the incubations, respective to the treatment. Calcification, photosynthesis, respiration and N_2 fixation rates were measured in 2 consecutive incubations. A total of 30 fragments were incubated with n = 15 for each CO_2 treatment level (ambient and high). Firstly, O_2 fluxes and calcification rates under treatment conditions (seawater of ambient or high CO_2) were quantified during the same incubation. Oxygen fluxes of the coral fragments were measured during light (PAR 110 ± 5 quanta µmol s^{-1} m^{-2}) and dark incubations (<2 h each to avoid supersaturation of O_2) in 250 ml glass chambers by constant logging of O_2 concentrations using O_2 optodes (Firesting, PyroScience Sensor Technology). Water samples of 50 ml were collected from each chamber before and after each incubation (light/dark) to measure calcification rates. All coral fragments were returned to the treatment aquaria of high or ambient CO_2, according to the treatment, for 24 h before start of the second incubation.

In the second incubation, the acetylene reduction technique was used to quantify N_2 fixation rates of the coral fragments (Hardy et al. 1968, Wilson et al. 2012). Coral fragments were incubated in 1 l glass chambers filled with 800 ml of treatment water (ambient or high CO_2 respectively), of which 10% (80 ml) was previously saturated with acetylene (C_2H_2) to improve equilibration in the chamber. Also, 10% (20 ml) of the 200 ml headspace was replaced with C_2H_2 gas after the chambers were sealed gastight. The incubation lasted for 22 h, starting with a 12 h dark phase followed by a 10 h light phase. During incubation, chambers were kept at a constant temperature of 26.0° ± 0.3°C. Gas samples of 1 ml were taken from the headspace after intervals of 0, 4, 12 and 22 h, and collected

in 2 ml glass vials previously filled with deionized water. Vials were stored frozen upside down until analysis to prevent any leakage from the septa.

Sample analyses

Respiration and gross photosynthesis rates were calculated from the incubation periods which showed linear changes in O_2 concentration. Changes in the total alkalinity of the water samples before and after the incubations were converted into calcification rates using the alkalinity anomaly technique (Chisholm & Gattuso 1991). Nitrogen fixation rates were calculated as ethylene (C_2H_4) evolution rates and not converted into actual fixation rates of N_2, as we acknowledge that there is an ongoing discussion about the correct conversion factor in the scientific community (Nohrstedt 1983, Wilson et al. 2012). C_2H_4 concentrations in the gas samples were quantified by gas chromatography (Varian 3800 with AL203/KCL 50 × 0.53 column and flame ionization detector). Changes in C_2H_4 concentration were converted into C_2H_4 evolution rates according to Breitbarth et al. (2004). N_2 fixation rates showed a distinct initial lag phase during the first 4 h of incubation. This is a common phenomenon during acetylene reduction assays (Zuberer & Silver 1978, Gallon & Hamadi 1984, Shashar et al. 1994b). Hence, N_2 fixation rates for the dark phase were calculated based on C_2H_4 concentration differences during the second time interval (4 to 12 h) without considering the first 4 h of incubation. Light N_2 fixation rates were calculated based on concentration differences between 12 and 22 h of incubation time.

Photosynthesis, respiration, calcification and N_2 fixation rates were corrected for seawater control (n = 6) signals and normalized to incubation time and coral surface area, which was quantified by advanced geometry (Naumann et al. 2009).

Data analysis

All statistical analyses were conducted with R version 3.0.2 (R Development Core Team 2013). Differences in N_2 fixation rates were analysed using generalized mixed effect linear models (GLMM) with gamma distribution and an inverse link function taking into account minor fluctuations in water temperatures during the incubations to increase the fit of the model. O_2 fluxes, calcification rates and the relationship of calcification with N_2 fixation rates were also analysed with generalized linear models (GLM) with gamma distribution and an inverse link function. To meet the assumptions of gamma distribution, O_2 fluxes, calcification and N_2 fixation rates were $(x + 1)$ transformed. All data were corrected for outliers using the Dixon test.

RESULTS

The seawater carbonate system following the manipulation of CO_2 concentrations showed significant differences. At a total alkalinity of 1784 ± 36 µmol kg^{-1} seawater, ambient CO_2 concentrations resulted in an aragonite saturation state (Ω Ar) of 1.9 ± 0.1 at a pH of 8.02, whereas high CO_2 concentrations showed an Ω Ar of 1.0 ± 0 at a pH of 7.71.

Short term exposure to high CO_2 concentrations revealed strong effects on the physiology of fragments of *Seriatopora hystrix* compared to the fragments incubated under ambient CO_2 concentrations. N_2 fixation activity (acetylene reduction) was variable, but measurable in all coral fragments. Rates were higher (3 to 4 times) in the light than in the dark, independently of the treatment applied ($\chi^2_{(1, N=30)} = 22.839$, $p << 0.001$). N_2 fixation rates ranged from 0.04–1.98 and

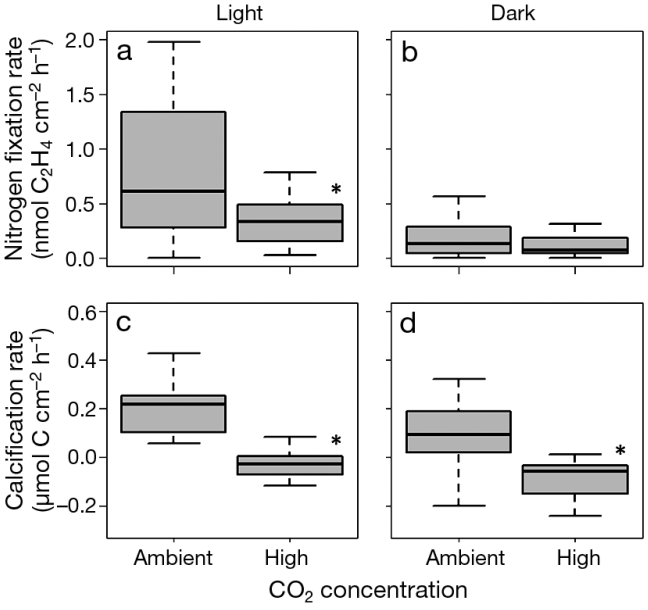

Fig. 1. (a,b) N_2 fixation rates (n = 15) and (c,d) calcification rates (n = 13) of *Seriatopora hystrix* under ambient and high CO_2 concentration treatments during the (a,c) light period and (b,d) dark period. N_2 fixation rates = ethylene (C_2H_4) production rates. Rates were corrected for seawater control and normalized to incubation time and coral surface area. Boxplot: mean, upper and lower quartiles; whiskers: data points within 1.5 times the interquartile range from the box. *$p < 0.01$ significantly different from each other

0.00–0.56 nmol C_2H_4 cm^{-2} h^{-1} during the light and dark period, respectively (Fig. 1a,b). High CO_2 levels caused a significant decline (53%) in the N_2 fixation rates of the coral holobiont in the light ($\chi^2_{(1, N=30)}$ = 6.8271, $p < 0.01$), reducing from 0.83 ± 0.16 (ambient) to 0.39 ± 0.09 nmol C_2H_4 cm^{-2} h^{-1} (high). This was not the case in the dark, because rates were too low to indicate any significant differences ($\chi^2_{(1, N=30)}$ = 0.8311, p = 0.36).

Overall, calcification rates ranged from −0.12 to 0.42 µmol C cm^{-2} h^{-1} in the light and −0.24 to 0.32 µmol C cm^{-2} h^{-1} in the dark period (Fig. 1c,d). Calcification rates showed a pronounced response to differences in CO_2 concentrations, significantly reducing under high CO_2 conditions compared to ambient CO_2 levels both in the light ($\chi^2_{(1, N=26)}$ = 26.651, $p << 0.001$) and in the dark period ($\chi^2_{(1, N=26)}$ = 4.55, $p << 0.001$). At ambient CO_2 concentrations, calcification rates were 0.20 ± 0.03 µmol C cm^{-2} h^{-1} in the light and 0.09 ± 0.04 µmol C cm^{-2} h^{-1}in the dark period. At high CO_2 concentrations, calcification rates were −0.01 ± 0.03 µmol C cm^{-2} h^{-1}in the light and −0.08 ± 0.02 µmol C cm^{-2} h^{-1}in the dark. Since both calcification and N_2 fixation decreased under the ocean acidification scenario, the relationship between these 2 processes was investigated (Fig. 2).

This revealed a positive exponential correlation of N_2 fixation activity and calcification rates in coral fragments incubated in the light ($\chi^2_{(1, N=25)}$ = 5.21, p = 0.02) as opposed to dark incubations (not shown), where the relationship was not significant ($\chi^2_{(1, N=25)}$ = 0.35, p = 0.55).

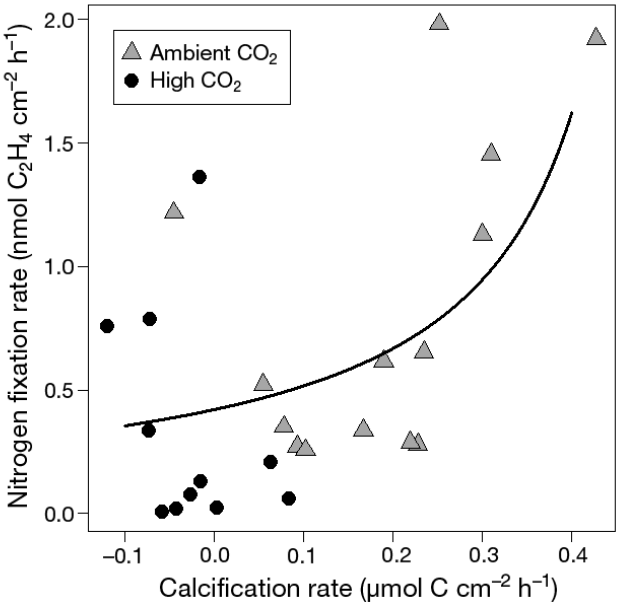

Fig. 2. Relationship of N_2 fixation rates and calcification rates of *Seriatopora hystrix* incubated in the light under high CO_2 (●) and ambient conditions (△). All rates were corrected for seawater controls and normalized to incubation time and coral surface area. Black curve: best-fitting model ($\chi^2_{(1, N=25)}$ = 5.21, p = 0.03, McFadden's R^2 = 0.862)

Differences in CO_2 concentrations had no significant effect on gross photosynthesis ($\chi^2_{(1, N=30)}$ = 0.01, p = 0.90) and respiration rates ($\chi^2_{(1, N=30)}$ = 0.18, p = 0.67) of the coral nubbins (not shown). Mean gross photosynthesis was 0.50 ± 0.04 µmol O_2 cm^{-2} h^{-1} under high CO_2 compared to 0.49 ± 0.05 µmol O_2 cm^{-2} h^{-1} under ambient CO_2 conditions. Respirations rates were −0.30 ± 0.03 and −0.28 ± 0.2 µmol O_2 cm^{-2} h^{-1} at high and low CO_2 conditions respectively.

DISCUSSION

This is the first study showing N_2 fixation associated with *Seriatopora hystrix* and demonstrating the effect of elevated CO_2 levels on N_2 fixation.

N_2 fixation has been described for several other coral species, with a pronounced variation between and within species (Williams et al. 1987, Shashar et al. 1994b, Lesser et al. 2007). To control for the intra-specific differences, manipulative experiments need to use individuals of identical genotype (Mascarelli & Bunkley-Williams 1999). All experiments in this study where conducted with coral colonies from the same colony. Thus the observed physiological changes can be referred back to treatment conditions.

N_2 fixation is an energy-intensive process (McNary & Burris 1962). Shashar et al. (1994a) found that N_2 fixation activity was inhibited in corals when photosynthesis was blocked with DCMU (3-[3,4-dichlorophenyl]-1,1-dimethylurea), but could be restored when glucose was added to the incubation water. This suggests that coral associated N_2 fixation strongly depends on photosynthetically fixed carbon to fulfil its energetic demands. In the present study, N_2 fixation rates were 3 to 4 times higher during the light incubations compared to the dark. This is likely explained by increased availability of fixed carbon by photosynthesis during the light phase. N_2 fixation occurred during times of net O_2 evolution, although O_2 is known to inhibit this process (Gallon 1981). There are different mechanisms by which N_2 fixation can take place at times of O_2 evolution (Gallon 1981). In coral reef sponges, for example, symbiotic non-heterocystous cyanobacteria, which depend on O_2 for their N_2 fixation, have been suggested to explain high N_2 fixation activity under aerobic conditions (Wilkinson & Fay 1979, Mohamed et al. 2008).

N_2 fixation rates were significantly reduced in the ocean acidification treatment compared to the ambient scenario in the light. Other studies reported an increase of N_2 fixation activity under elevated CO_2 conditions for planktonic cyanobacteria due to increased photosynthetic carbon fixation by overcoming CO_2 limitation (Hutchins et al. 2007, Garcia et al. 2013). This may be the case for planktonic autotrophic diazotrophs, but CO_2 limitation is unlikely to occur in the *S. hystrix* holobiont due to respiration by the coral host. Reduced N_2 fixation rates under elevated CO_2 concentrations have only been described in the planktonic cyanobacterium *Trichodesmium* in combination with low iron availability (Shi et al. 2012). Since the experiments carried out in the present study took place in laboratory conditions, it is unlikely that iron limitation caused the lowering of fixation rates in the short time span of the experiment described in the present study. Hence, there has to be another cause for the effects observed. Along with N_2 fixation, calcification of *S. hystrix* was significantly reduced during both light and dark periods. The significant positive correlation between both processes during the light may suggest an indirect linkage of the 2 processes in the holobiont.

The reduced calcification rates are in good agreement with previous studies reporting similar effects under low pH conditions due to lowered aragonite saturation state (Orr et al. 2005, Anthony et al. 2008, Kleypas & Yates 2009). Since N_2 fixation and calcification are energy-intensive mechanisms, they likely compete for energy within the coral holobiont. The lowering in the aragonite saturation state makes the calcification process more energy consuming (Marubini et al. 2001, Hohn & Merico 2012). Since gross and net photosynthesis were not significantly different between treatments, the increased energy demand by calcification at high CO_2 conditions may create an energy deficit in the coral holobiont. Subsequently, this may also reduce the energy available for heterotrophic diazotrophs in the coral tissue, thereby explaining the decrease in N_2 fixation activity at high CO_2 conditions. Although Anthony et al. (2008) reported a loss of coral productivity at lower seawater pH during long term experiments, there was no effect of elevated CO_2 on photosynthesis and respiration of the fragments used in the present study, probably due to the short time span of the incubations. It is hence likely that the described long term drop in productivity will amplify the effects of ocean acidification on N_2 fixation and calcification even more. This is the first evidence that coral associated N_2 fixation can be affected by ocean acidification. The observed decline in N_2 fixation may result in N starvation for both the coral and *Symbiodinium* spp. Together with a reduced skeletal growth, this suggests a negative feedback loop for the productivity of the holobiont. The reduction in N_2 fixation may thus exacerbate negative long-term effects of ocean acidification for coral reef functioning. Finally, these findings highlight the importance of N_2 fixation as a key process for understanding the response of the coral holobiont to environmental stressors such as ocean acidification. To improve the understanding of interactions between diazotrophs, *Symbiodinium* spp. and the coral host, an interdisciplinary approach is needed, combining ecological and microbiological aspects.

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