Coral calcification responds to seawater acidification: a working hypothesis towards a physiological mechanism

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Abstract The decrease in the saturation state of seawater, Ω , following seawater acidification, is believed to be the main factor leading to a decrease in the calcification of marine organisms. To provide a physiological explanation for this phenomenon, the effect of seawater acidification was studied on the calcification and photosynthesis of the scleractinian tropical coral *Stylophora pistillata*. Coral nubbins were incubated for 8 days at three different pH (7.6, 8.0, and 8.2). To differentiate between the effects of the various components of the carbonate chemistry (pH, CO_3^{2-} , HCO_3^- , CO_2 , Ω), tanks were also maintained under similar pH, but with 2-mM HCO_3^- added to the seawater. The addition of 2-mM bicarbonate significantly increased the photosynthesis in *S. pistillata*, suggesting carbon-limited conditions. Conversely, photosynthesis was insensitive to changes in pH and pCO_2 . Seawater acidification decreased coral calcification by ca. 0.1-mg $\text{CaCO}_3 \text{ g}^{-1} \text{ d}^{-1}$ for a decrease of 0.1 pH units. This correlation suggested that seawater acidification affected coral calcification by decreasing the availability of the CO_3^{2-} substrate for calcification. However, the decrease in coral

calcification could also be attributed either to a decrease in extra- or intracellular pH or to a change in the buffering capacity of the medium, impairing supply of CO_3^{2-} from HCO_3^- .

Keywords Calcification · Biomineralization · Carbon supply · Calicoblastic cells · Buffering capacity · Saturation state of aragonite

Introduction

The deposition of the calcium carbonate skeleton by scleractinian corals is a highly organized extracellular process. Skeletogenesis is not merely a product of inorganic precipitation out of a super-saturated solution, rather it is regulated by a process that combines organic matrix and mineral depositions in seasonal to diurnal growth layers (Barnes and Lough 1993; Cuif et al. 1999; Cohen and McConnaughey 2003; Allemand et al. 2004; Cuif and Dauphin 2005; Tambutté et al. 2007). Calcification rate is controlled by the calicoblastic epithelium, which provides the peri-crystalline fluid (i.e., the fluid filling the space between epithelium and skeleton) with all necessary factors for calcification. The calicoblastic epithelium secretes the organic matrix (Johnston 1980; Allemand et al. 1998; Puverel et al. 2005; Tambutté et al. 2007), and transports calcium ions (Tambutté et al. 1996; Marshall 1996) and dissolved inorganic carbon (DIC) (Goreau 1961; Furla et al. 2000). The experimental inhibition of any of these processes has been shown to inhibit calcification (Allemand et al. 2004).

Inorganic precipitation of calcium carbonate was also found to be proportional to Ω , the saturation state of seawater, which is equal to $[\text{Ca}^{2+}] \times [\text{CO}_3^{2-}]/K$, where K is

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the apparent solubility constant for a particular mineral phase of CaCO_3 . It is therefore largely assumed that Ω also controls coral calcification at the organismal level (Smith and Buddemeier 1992; Gattuso et al. 1998; Marubini et al. 2001, 2003; see review Raven et al. 2005). A solution with a low Ω is not energetically favourable for CaCO_3 precipitation (Simkiss 1976), and warrants concern for reef health worldwide because Ω has already decreased over the last century from 5.3 to 4.4 and will keep on decreasing as atmospheric pCO_2 increases (Kleypas et al. 1999, 2005; Orr et al. 2005). Such increase in pCO_2 has already led to a decrease in pH of about 0.1 pH unit during the last century (IPCC 2007), resulting in a decrease in $[\text{CO}_3^{2-}]$ and thus Ω . Numerous laboratory experiments have demonstrated that a decrease in seawater Ω , by manipulating either calcium or DIC concentration, induced a correlative 10–30% decrease in coral calcification (Gattuso et al. 1998; Leclercq et al. 2002; Marubini et al. 2003; Ohde and Hossain 2004; Schneider and Erez 2006; Fine and Tchernov 2007).

One of the main questions that remains to be answered is how can changes in seawater lead to consistent and significant differences in calcification when the magnitude of these changes is much smaller than the large fluctuations in Ω , pH, or DIC content and speciation that occur within the coelenteric environment between light and dark conditions (Furla et al. 1998, 2000; Al-Horani et al. 2003)? Indeed Furla et al. (1998) showed that the coelenteric pH fluctuates from 7.4 at night to 8.9 during the day, i.e., a ΔpH far larger than that induced by ocean acidification. Furthermore, while two studies performed in the medium term (>1 week) have shown an enhancement of coral growth after an increase in DIC (Marubini and Thake 1999; Herfort et al. 2008), a different result has been found with experiments made on a scale of a few hours, which showed that coral growth was saturated at $\sim 1\text{-mM}$ DIC (Furla et al. 2000). A potential explanation for these contrasting results may be differences in the period of incubation in DIC-enriched medium, perhaps inducing acclimatization of corals.

Since a decrease in Ω is always linked to a decrease in pH and in CO_3^{2-} as well as an increase in CO_2 and HCO_3^- , it is difficult to decipher which parameter, CO_3^{2-} , pH, or pCO_2 , is responsible for the observed decrease in calcification. Indeed, decreased pH can change both the availability of nutrients (especially metals, Zeebe and Wolf-Gladrow 2001), and lead to the acidification of body fluids in marine animals, indirectly affecting their growth; increased CO_2 concentrations also alter the level of bicarbonate used both in coral photosynthesis and calcification (Langdon and Atkinson 2005).

By doubling the bicarbonate concentration in seawater, it is possible, however, to dissociate the effects of pH and Ω . If the decrease in calcification is related to Ω and not to

pH, corals would be expected to grow faster under high Ω values. Conversely, if coral physiology and cellular processes are governed by pH, then, corals grown at the same pH should present the same growth rate, independent of Ω . To test this hypothesis, an 8-day experiment was performed maintaining the branching coral *Stylophora pistillata* in three different pH and two concentrations of total DIC for each pH. The effect of the changing seawater chemistry was monitored in both rates of photosynthesis and calcification, since the coupling between these two processes, which depend on the same substrate, HCO_3^- (see review by Allemand et al. 2004), is well known (McConnaughey and Whelan 1997). If increased CO_2 concentrations enhance photosynthesis (Goiran et al. 1996), this would be expected to have an effect on the rates of calcification.

Materials and methods

Colonies of *S. pistillata* were used in this study because its physiology is well known (Muscatine et al. 1989; Tambutté et al. 1996; Shick et al. 2005; Franklin et al. 2006; Stambler and Shashar 2007; Mass et al. 2007). Colonies were sampled in the Gulf of Aqaba in surface waters and maintained for 5 months under culture conditions. Similar sized tips, hereafter called nubbins (2.5-cm length for calcification, 1-cm length for photosynthesis), were cut from parent colonies using bone cutters. The nubbins were hung by a nylon thread from a supporting structure above the culture aquaria, and were left to recover and grow in the same conditions as the parent colonies for 2 months prior to the start of the experiment. Nubbins were not fed. On average the tissue had already covered the cut surface within the first 2 weeks. At the start of the experiment, nubbins for calcification weighed on average 1.82 g (min 0.45, max 3.91 g).

Six 20-l aquaria were used to each hold 11 coral nubbins for calcification, and 5 for photosynthesis. Mediterranean seawater from a depth of 50 m was pumped directly into each aquarium at a rate of $1,000\text{ ml min}^{-1}$ and the overflow was drained back to the sea. Each tank was fitted with a temperature controller (ElliWell PC902/T), a sensor and a heater (RENA 500W). Temperature was maintained constant at $26.5 \pm 0.3^\circ\text{C}$ (mean $\pm$ SD). Light was provided 12 h d^{-1} by two overhead metal halide lamps (OSRAM HIQ-T 400W bulbs). Total photosynthetically available radiation (PAR) was $300 \pm 40\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ as measured in situ with an underwater spherical (4π) light meter (LICOR 196SA).

The incoming seawater presented a very constant chemistry during the whole experimental period: salinity 38.3, pH = 7.95, total Alkalinity = $2,500\text{ }\mu\text{eq kg}^{-1}$, and was used directly for one treatment. All 5 other chemical

Table 1 Carbonate chemistry for each experimental condition

Parameter	BA-7.6	BA-8.0	BA-8.2	BE-7.6	BE-8.0	BE-8.2
TA ($\mu\text{eq kg}^{-1}$)	2,314 $\pm$ 22	2,544 $\pm$ 27	2,749 $\pm$ 14	3,905 $\pm$ 138	4,342 $\pm$ 110	4,657 $\pm$ 163
pH _{sw}	7.58 $\pm$ 0.050	7.97 $\pm$ 0.022	8.19 $\pm$ 0.017	7.56 $\pm$ 0.043	7.95 $\pm$ 0.029	8.16 $\pm$ 0.020
TCO ₂ ($\mu\text{mol kg}^{-1}$)	2,204	2,224	2,243	3,762	3,881	3,927
pCO ₂ (μatm)	1,323	515	293	2,367	945	556
CO ₃ ²⁻ ($\mu\text{mol kg}^{-1}$)	99.1	232.5	364.8	161.7	389.1	602.6
HCO ₃ ⁻ ($\mu\text{mol kg}^{-1}$)	2,069	1,978	1,870	3,537	3,466	3,310
CO ₂ ($\mu\text{mol kg}^{-1}$)	35.5	13.8	7.9	63.5	25.3	14.9
β (mM pH units ⁻¹)	3.090 $\times 10^{-4}$	1.490 $\times 10^{-4}$	0.994 $\times 10^{-4}$	5.43 $\times 10^{-4}$	2.66 $\times 10^{-4}$	1.80 $\times 10^{-4}$
$\Omega_{\text{aragonite}}$	1.54	3.62	5.68	2.52	6.06	9.39

Total alkalinity (TA) and pH data are means ($\pm$ SD) over the whole experimental period. The rest are calculated from TA and pH using the apparent dissociation constants of Mehrbach et al. (1973) for carbonic acid. The aragonite saturation state was calculated according to Mucci (1983). BA = bicarbonate-ambient, BE: bicarbonate-enriched seawater

treatments (see Table 1) were achieved by continuously dripping the required amounts of NaHCO₃, HCl or NaOH into each aquarium with a peristaltic pump from solutions made up daily. Treatments were called BA-7.6, 8.0 and 8.2 for bicarbonate-ambient seawater (2 mM) at the three pH tested, and BE-7.6, 8.0, and 8.2 for bicarbonate-enriched seawater (4 mM) at the same three pHs. Across the six treatments, Ω_{arag} varied 6-fold from 1.54 (for BA-7.6) to 9.39 (for BE-8.2). Total alkalinity was measured potentiometrically with an automated titrator (Metler DL70) and computed using the Gran equation (DOE 1994). Samples were taken three times a week. pH was measured twice a day on the seawater scale. The components of the carbonate system (pCO₂, HCO₃⁻, CO₃²⁻) were calculated from TA and pH using the apparent dissociation constants of Mehrbach et al. (1973) for carbonic acid. Ca²⁺ was determined from salinity and temperature. The aragonite saturation state was calculated according to Mucci (1983). Changes in osmolarity, due to addition of NaHCO₃, HCl or NaOH, were calculated and found to be negligible. The buffering capacity (β , expressed as mM. pH units⁻¹) of the medium was also considered as the coefficient dB/dpH, where dB is the increment of strong base (acid) added, and dpH the resultant increment of pH. β was calculated from the equation $\beta = \ln 10 \cdot K_a \cdot [\text{H}^+] \cdot \text{TA} / (K_a \cdot [\text{H}^+])^2$, where K_a is the equilibrium constant of the couple (CO₂ + H₂O)/HCO₃⁻ (pK_a = 6.4), TA the total alkalinity, and [H⁺] the concentration of H⁺ (Truchot 1987).

Rates of photosynthesis were measured at the end of the 8-days of incubation as described in Houlbrèque et al. (2003). Briefly, nubbins were incubated in a 15-ml glass thermostated chamber, filled with the different incubation media, continuously stirred and containing a Strathkelvin 928[®] oxygen electrode (Clark-type electrode). Electrodes were calibrated against air-saturated (100%) and sodium dithionite saturated (zero oxygen) incubation media (Houlbrèque

et al. 2003). Samples were allowed to acclimate until polyps were expanded. Changes in dissolved oxygen concentrations were monitored on a computer at the culture irradiance and in the dark. Rates were estimated by regressing oxygen data against time, taking into account the seawater volume in the chamber. At the end of the measurements, samples were frozen at -80°C for chlorophyll *a* determination.

Coral skeletal weight was measured at the beginning and at the end of the 8-day experiment by buoyant weighing (Davies 1989). Corals were handled only by their nylon thread and suspended below a HERAUS precision balance (0.1 mg). The tissue of *S. pistillata* accounted for ca. $1 \pm 0.5\%$ of the total buoyant weight, and a small correction was therefore made to the results, as in Davies (1989). Growth rate was then calculated on a daily basis and standardized to initial skeletal weight.

The effect of the water chemistry was tested using a two-way ANOVA using StatView software. Results were considered significantly different for values of $P < 0.05$.

Results

pH treatments were similar in bicarbonate-ambient and bicarbonate-enriched seawater (low pH = 7.58 and 7.56; medium pH = 7.97 and 7.95; high pH = 8.19 and 8.16). pH was maintained constant (maximum variability = 0.05) throughout the experiment. There was no appreciable effect of coral metabolism on seawater chemistry due to the short residence time in the aquaria. Table 1 presents all other parameters of the carbon chemistry in the aquaria.

Protein and chlorophyll *a* concentrations did not differ between treatments after 8 days (ca. 6 g and 80 mg g⁻¹, respectively). Figure 1 shows the rates of photosynthesis measured in the experimental tanks. There was no effect of pH. However, rates of photosynthesis were significantly

Fig. 1 Effect of seawater acidification on the photosynthetic rate of nubbins of *Stylophora pistillata*. Photosynthetic rates were measured after 8 days of incubation in bicarbonate-ambient (BA, 2 mM) and bicarbonate-enriched (BE, 4 mM) at 3 different pH: 7.6, 8.0, and 8.2 (means $\pm$ SE)

Fig. 2 Effect of seawater acidification on the growth rate of nubbins of *Stylophora pistillata*. Growth rates (G) were measured after 8 days of incubation in bicarbonate-ambient (BA, 2 mM) and bicarbonate-enriched (BE, 4 mM) at 3 different pH: 7.6, 8.0, and 8.2 (means $\pm$ SE)

($F_{1,50} = 46.36$, $P < 0.001$) higher (by ca. 70%) in the bicarbonate-enriched compared to bicarbonate-ambient treatments (Fig. 1).

Coral calcification also varied between treatments (Fig. 2). It was lowest in the BA-7.6 condition ($14.7 \text{ mg CaCO}_3 \text{ g}^{-1} \text{ d}^{-1}$) and maximum in the BE-8 and 8.2 treatments (24.6 and $24.1 \text{ mg CaCO}_3 \text{ g}^{-1} \text{ d}^{-1}$). There was an effect of both pH ($F_{2,60} = 12.7$, $P < 0.0001$) and DIC ($F_{1,60} = 23.5$, $P < 0.0001$) on calcification with no interaction. On average for the same pH, corals in the bicarbonate-enriched seawater grew 27% faster (range 15–39%) than in bicarbonate-ambient seawater. Growth rates were also plotted against the different parameters

involved in the DIC equilibrium, i.e., HCO_3^- , CO_3^{2-} , CO_2 and pH (Fig. 3; Table 2). For CO_3^{2-} or Ω_{arag} there was no significant relationship with growth rate for the data of the separate treatments (bicarbonate-ambient or bicarbonate-enriched) but using all data points, there was a positive relationship in both cases (Table 2, Fig. 3d, e) which tended towards an asymptote as CO_3^{2-} or Ω_{arag} increased. For all other parameters except HCO_3^- , growth was linearly correlated for each treatment separately (Table 2, Fig. 3a–c and f).

Discussion

The present study investigated the medium term (1-week incubation) effect of seawater acidification on calcification and photosynthesis of the branched coral, *S. pistillata*. Since acidification alters DIC equilibrium, experiments were performed at normal and double concentrations of $[\text{HCO}_3^-]$ to discriminate between a pH or Ω (or CO_3^{2-}) effect on calcification.

Effect of DIC concentration on photosynthesis

The results show that the addition of 2-mM bicarbonate in the medium doubled the photosynthesis in *S. pistillata*, suggesting carbon-limited conditions in this experiment. This is in agreement with the observations of Weis (1993), who demonstrated a bell-shape dependence of the net photosynthetic rate for the sea anemone *Aiptasia pulchella* on DIC concentrations, with maximal rates obtained at ca. 5-mM DIC. A similar carbon-limitation has also been shown for the corals *Porites porites* and *Acropora* sp. with an optimal concentration of 6-mM DIC (Herfort et al. 2008). Flow experiments or stable isotope measurements using corals have also suggested carbon-limitation for photosynthesis (Dennison and Barnes 1988; Muscatine et al. 1989; Lesser et al. 1994; Kühl et al. 1995), but these findings contrast with numerous other observations. Burris et al. (1983) and Schneider and Erez (2006) failed to enhance the rates of photosynthesis of *S. pistillata* and *Acropora eurytoma* by the addition of bicarbonate, up to concentrations of ca. 4.3 and 2.7 mM, respectively. Goiran et al. (1996) also demonstrated that the photosynthesis of *Galaxea fascicularis* was saturated at around 1.5-mM bicarbonate, either at constant pH or pCO_2 . Finally, zooxanthellae photosynthesis did not change with a change in CO_2 and pH (Leclercq et al. 2002; Langdon et al. 2003). The differences in these studies may be a consequence of the different incubation lengths used. Indeed, all the experiments on corals showing no DIC enhancement of photosynthesis were conducted on a rather short time scale (generally a few hours), while in the present experiment,

Fig. 3 Growth rate (G) of nubbins of *Stylophora pistillata* as a function of (a) HCO_3^- , (b) dissolved CO_2 , (c) pH, (d) CO_3^{2-} , (e) Ω , and (f) β (means $\pm$ SE). Experiments were carried out in bicarbonate-ambient (black symbols) or bicarbonate-enriched (white symbols) seawater

Table 2 Statistical models testing the shape and significance of the relationships of growth (G) to water parameters and their interactions (as shown in Fig. 3)

Relationship	Equation	R^2
<i>G versus CO_2 concentrations</i>		
Bicarbonate-ambient	$y = -0.204 x + 21.69$	0.89 ($P = 0.006$)
Bicarbonate-enriched	$y = -0.133 x + 27.48$	0.99 ($P = <0.001$)
<i>G versus pH</i>		
Bicarbonate-ambient	$y = 10.071 x - 62.10$	0.94 ($P = 0.003$)
Bicarbonate-enriched	$y = 10.893 x - 63.55$	0.96 ($P = <0.001$)
<i>G versus CO_3^{2-} concentrations</i>		
All points	$y = 5.744 \ln(x) - 11.64$	0.87 ($P < 0.001$)
<i>G versus Ω</i>		
All points	$y = 5.739 \ln(x) - 12.28$	0.87 ($P < 0.001$)
<i>G versus β</i>		
Bicarbonate-ambient	$y = -5.381 \ln(x) - 29.05$	0.96 ($P = 0.002$)
Bicarbonate-enriched	$y = -6.122 \ln(x) - 26.92$	0.98 ($P = 0.001$)

corals were maintained for more than 1 week in the enriched DIC medium. The longer term increased availability of the substrate may have led to an up-regulation of either carbon transport mechanisms or of the photosynthetic system.

The present results also demonstrate that photosynthesis was insensitive to pH and pCO_2 . Indeed for a given DIC concentration, pH and CO_2 had no significant effect on the rates of net photosynthesis, although this pH decreased by up to 0.6 units, while $[\text{CO}_2]$ increased by 27.6 μM in bicarbonate-ambient seawater and by 48.6 μM in bicarbonate-enriched seawater (i.e., ca. 4.5-fold increase in CO_2 concentration). The lack of pH effect on photosynthesis was also observed by Goiran et al. (1996) and more recently by Schneider and Erez (2006). However, Goiran et al. (1996) showed that photosynthesis measured in a closed system was enhanced by an increase in dissolved CO_2 at constant DIC, suggesting that CO_2 supply to photosynthesis may be limiting while total DIC is at saturating concentration. This latter result is however surprising when compared to the present one, where a 4.5-fold increase in dissolved CO_2 did not cause any enhancement in O_2 production, while a 2-fold increase in total DIC led to almost a

doubling in O_2 production. This suggests that the permeability of zooxanthellae and/or the peri-symbiotic membrane to CO_2 is low. Due to this low permeability, the host can easily control the carbon supply to zooxanthellae using membrane carriers.

Effect of change of DIC equilibrium on calcification

It is assumed that global change-induced ocean acidification is leading to a global decrease in marine calcification (see review by Raven et al. 2005). The results of the present study confirm that seawater acidification leads to a decrease in the growth rate of a tropical coral, *S. pistillata*. The magnitude of this decrease was about 1-mg $CaCO_3$ g^{-1} d^{-1} for each 0.1 pH unit.

However, the results also showed that a doubling of the seawater bicarbonate concentration lead to an increase in mean growth of 27%, independent of pH conditions, suggesting that calcification was C-limited. The smallest increase was at a pH of 8.2 (15% compared to 28 and 39% at 7.6 and 8, respectively). Marubini and Thake (1999) have previously demonstrated that the addition of 2-mM bicarbonate increased growth rates as well as linear extension rates, and that the response was quick and sustained. More recently, Herfort et al. (2008) also found that the calcification rate of the corals *Porites porites* and *Acropora* sp. maintained at pH 8.2 increased with an increase in bicarbonate concentration, up to a total of 6- and 8-mM DIC, respectively. These findings contradict the HCO_3^- -dependence curve published by Furla et al. (2000), who showed that calcification was saturated at a concentration of ca. 1-mM HCO_3^- . Discrepancies between these studies may again be due to the length of the acclimatization process: between 8 h and 32 days for experiments in which a change in calcification rate was observed (Marubini and Take 1999; Herfort et al. 2008; present study) and <180 min when no change was recorded (Furla et al. 2000). A similar up-regulation of proteins involved in C-transport, as suggested for photosynthesis, may explain this result, and implies that corals are able to adapt to change in DIC concentration. Another explanation could be the enhancement of calcification by an enhancement of photosynthetic products. Indeed, both photosynthesis and calcification were significantly increased by the addition of bicarbonate, and it is well known that photosynthesis supplies some precursors of the skeletal organic matrix (see Tambutté et al. 2007 for a review). However, the two mechanisms were not correlated (see below) and there was no proportionality either, since photosynthesis was increased by a factor of ca. 2, while calcification was only increased by a factor of ca. 1.4.

To determine if the decrease in calcification observed upon acidification was mediated by the direct effect of one

particular parameter, relationships were established between skeletal growth and all parameters of the carbon equilibrium. When plotting growth versus the entire set of data for each parameter, a clear relationship was only obtained with $[CO_3^{2-}]$ and Ω_{arag} . However, when data were considered separately for each medium (bicarbonate-ambient and bicarbonate-enriched), linear relationships were observed with all parameters, except bicarbonate (Fig. 3). The lowest concentration of bicarbonate used in the present experiment (1.87 mM) was almost 2-fold higher than the concentration for which Furla et al. (2000) observed a saturation of calcification (1 mM). Since the increase in growth was non-linear, it is possible that bicarbonate ion concentrations above 1 mM are not limiting for calcification, and the supply of carbonate ions for calcification is based upon a simple equilibrium.

Examining the findings that coral growth was correlated with the three parameters (pH, CO_3^{2-} and CO_2) leads to five hypotheses:

(1) *Seawater acidification affects coral calcification through a decrease in photosynthesis.* This hypothesis is not supported since calcification and photosynthesis were not correlated (Figs. 1 and 2). Indeed, while calcification was dependent on CO_3^{2-} concentration, photosynthesis was not, suggesting some uncoupling of the processes, as already observed by Schneider and Erez (2006). This uncoupling refutes a possible competition for DIC between calcification and photosynthesis, as suggested by Langdon and Atkinson (2005).

(2) *Seawater acidification affects coral calcification through a decrease in carbonate concentration.* This hypothesis is supported by the significant correlation between growth and CO_3^{2-} concentration and is in agreement with previous studies performed at the organism (Marubini and Atkinson 1999; Marubini et al. 2001, 2003; Schneider and Erez 2006) and community (Langdon 2000; Langdon et al. 2003; Leclercq et al. 2000) levels. While in most studies, a linear relationship between coral calcification and $[CO_3^{2-}]$ was observed (Langdon and Atkinson 2005; Schneider and Erez 2006), this relationship was hyperbolic in the present study. This discrepancy is probably related to the different range of carbonate concentrations tested; indeed, the range used by Schneider and Erez (2006), for example, ca. 200 to <500 μM exactly fits within the linear portion of the plot depicted in Fig. 3d. Such a relationship suggests a Michaelis-Menten dependence of coral calcification upon carbonate, or in other words an enzymatic-linked process.

Since increases in $[Ca^{2+}]$ or $[CO_3^{2-}]$ have the same effect on calcification (Gattuso et al. 1998; Langdon 2000), it has been hypothesized that calcification is directly affected by the ion concentration product $[Ca^{2+}] \times [CO_3^{2-}]$ and not by the change in the carbonate chemistry per se (Kleypas et al.

1999). A good correlation was also observed in this experiment when coral growth was plotted against Ω_{arag} (Fig. 3e), the curve being similar to the one obtained with CO_3^{2-} (Fig. 3d). However, the correlation between ambient Ω and calcification rates is not always obvious (McConnaughey et al. 2000). Indeed, although the coral skeleton is extracellular, it is isolated from seawater by two to four tissue layers (Johnston 1980; Tambutté et al. 2007) and would probably never directly experience seawater Ω (McConnaughey et al. 2000). How then could changes in carbonate ion concentrations interfere with coral calcification?

If the carbonate ion is the direct carbon source for calcification, it can be supplied either through passive equilibrium between the coelenteron and the peri-crystalline fluid or by a specific transepithelial transport through a CO_3^{2-} carrier. Although still under debate, the passive transepithelial transport is not supported by the numerous arguments suggesting that the calcicoblastic epithelium behaves as a tight epithelium (for review see Cohen and McConnaughey 2003; Allemand et al. 2004). Concerning the CO_3^{2-} carrier hypothesis, no specific CO_3^{2-} carrier has been described until now in the literature, leaving only the possibility that CO_3^{2-} binds to an anion carrier, such as the electrogenic $\text{Na}^+/\text{HCO}_3^-$ co-transporters or the sulphate anion transporters in place of HCO_3^- (see for review Boron 2001; Casey 2006). Furla et al. (2000) were unable to provide evidence that CO_3^{2-} plays a direct role in coral calcification. Moreover, assuming that the intracellular pH (pHi) of the calcicoblastic cells is about 7.4 (a typical value for marine invertebrate cells, Payan et al. 1983), the speciation of DIC would lead to a small amount of CO_3^{2-} compared to HCO_3^- thus precluding any direct involvement of this ion. The correlation with carbonate does not mean that this ion is directly used for calcification.

(3) *Seawater acidification affects coral calcification through an inhibition induced by high CO_2 concentrations.* Upon hydration, CO_2 may lead to acidification of the medium and then alteration of the biological processes. The intracellular CO_2 concentration is unknown, but can be estimated taking into account an intracellular bicarbonate concentration of about 3.7 mM (Furla et al. 2000), and a pHi value of 7.4 for marine organisms (Payan et al. 1983). From these assumptions, the intracellular CO_2 concentration, calculated using the Henderson-Hasselbalch equation, $\text{pH} = \text{pK} + \log [\text{HCO}_3^-]/[\text{CO}_2]$ with $\text{pK} = 6.1$ (Truchot 1987) would be equal to ca. 180 μM , a value below that reported for vertebrate cells which are in the range 5–15 mM for an extracellular P_{CO_2} of about 5% (Stinner et al. 1994). Therefore a CO_2 increase from 10 to 35.5 μM (i.e., a Δ of 25.5 μM as in the present study) would decrease pHi by about 0.04 pH units, a value within the range of natural variation experienced by the organism, and therefore insufficient to modify cell physiology as already

suggested in plant cells (Bown 1985). Unfortunately, it is impossible to make such calculation for the peri-crystalline fluid, since the HCO_3^- concentration is unknown, but as this concentration is likely higher than in cells (Allemand et al. 2004), the pH decrease should be even smaller.

(4) *Seawater acidification affects coral calcification through an inhibition induced by decreasing pH.* The range of seawater pH, or extracellular pH (pHe) used in the present study or in most of the above cited literature is large (≈ 0.6 units), but remains in the same range as those observed during a daily cycle in coral tissue (Kühl et al. 1995; Al-Horani et al. 2003) or within the ecosystem (Suzuki et al. 1995), suggesting that coral cells are already adapted to small variations in external pH.

Cells regulate their internal pH (pHi) by maintaining a difference compared with the extracellular medium (Roos and Boron 1981). Consequently, when extracellular pH varies, pHi also varies, with the difference depending on the intracellular buffering capacity. In general, internal variations are one half of the external variations (Tufts and Boutilier 1989; Wahl et al. 1996). A similar assumption can be made for transepithelial pH between coelenteric cavity and peri-crystalline fluid. In the present study, a ΔpHe of ca. 0.6 pH units led to a 30–21% decrease in calcification at bicarbonate-ambient and bicarbonate-enriched concentration, respectively. Even if this ΔpH is lowered by the buffering capacity of the medium, we should expect a significant change, either in the pHi of the calcicoblastic cells or of the peri-crystalline fluid, leading to a decrease in the rate of CO_2 hydration and CO_3^{2-} formation. Variation of pHe/pHi may also affect protonation of amino acid residues such as histidine thus leading to conformational change in protein coupled to a change in their activity (Jiang et al. 2005).

(5) *Seawater acidification affects coral calcification indirectly through a change in the buffering capacity of the coelenteric space or of the peri-crystalline fluid.* The buffering capacity (β , expressed as mM pH units^{-1}) is defined as:

$$\beta = \text{dB}/\text{dpH}$$

where dB is the amount of base (or H^+) added to the solution and dpH is the change in pH of the solution due to that base (or acid) addition. When β increases, pH change induced by a given amount of base (or acid) decreases. An inverse relationship was found between coral calcification and β (Fig. 3f), with slopes and absolute values depending on total DIC. These data therefore suggest that low buffering capacity of the media is associated with high rates of coral calcification. Allemand et al. (2007) reported the same observation in fish otoliths during metabolic acidosis, where a low buffering capacity was reported close to a site of high calcification rate. We may hypothesize that a high β may prevent the equilibration between HCO_3^- and CO_3^{2-} ,

since this equilibrium is dependent upon pH, either in the coelenteron or in the peri-crystalline fluid, thus stopping the carbonate supply to calcification.

In conclusion, this study confirms that global change-induced seawater acidification may lead to a decrease of tropical coral growth calcification. While the biological mechanism underlying this decrease remains to be elucidated, it is proposed that this effect is either mediated by a decrease in carbonate, in pH, or by an alteration of the internal buffering system leading to a disruption of carbon supply to calcification rather than by a direct effect of CO₂ or a change of HCO₃⁻ concentration. Results showed that the negative effect of acidification may be counteracted by increasing the bicarbonate concentration of seawater, resulting in an increase in the carbonate concentration.

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