Effects of variations in carbonate chemistry on the calcification rates of *Madracis auretenra* (= *Madracis mirabilis* sensu Wells, 1973): bicarbonate concentrations best predict calcification rates

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Abstract

Physiological data and models of coral calcification indicate that corals utilize a combination of seawater bicarbonate and (mainly) respiratory CO$_2$ for calcification, not seawater carbonate. However, a number of investigators are attributing observed negative effects of experimental seawater acidification by CO$_2$ or hydrochloric acid additions to a reduction in seawater carbonate ion concentration and thus aragonite saturation state. Thus, there is a discrepancy between the physiological and geochemical views of coral biomineralization. Furthermore, not all calcifying organisms respond negatively to decreased pH or saturation state. Together, these discrepancies suggest that other physiological mechanisms, such as a direct effect of reduced pH on calcium or bicarbonate ion transport and/or variable ability to regulate internal pH, are responsible for the variability in reported experimental effects of acidification on calcification. To distinguish the effects of pH, carbonate concentration and bicarbonate concentration on coral calcification, incubations were performed with the coral *Madracis auretenra* (= *Madracis mirabilis* sensu Wells, 1973) in modified seawater chemistries. Carbonate parameters were manipulated to isolate the effects of each parameter more effectively than in previous studies, with a total of six different chemistries. Among treatment differences were highly significant. The corals responded strongly to variation in bicarbonate concentration, but not consistently to carbonate concentration, aragonite saturation state or pH. Corals calcified at normal or elevated rates under low pH (7.6–7.8) when the seawater bicarbonate concentrations were above 1800 μM. Conversely, corals incubated at normal pH had low calcification rates if the bicarbonate concentration was lowered. These results demonstrate that coral responses to ocean acidification are more diverse than currently thought, and question the reliability of using carbonate concentration or aragonite saturation state as the sole predictor of the effects of ocean acidification on coral calcification.

Keywords: aragonite saturation state, biomineralization, coral calcification, *Madracis auretenra*, Ocean acidification, seawater pH

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Introduction

Ocean acidification is the term used to describe the phenomenon of reduced seawater pH resulting from the absorption of atmospheric CO$_2$ from anthropogenic sources. Although the process increases seawater total dissolved CO$_2$ (DIC), pCO$_2$ and HCO$_3^-$ concentration [HCO$_3^-$], it lowers both pH and CO$_3^{2-}$ concentration [CO$_3^{2-}$]. The decreased [CO$_3^{2-}$] (at constant [Ca$^{2+}$]) causes a reduction in the saturation state of aragonite (Ωarag), defined as the product of Ca$^{2+}$ and CO$_3^{2-}$ concentrations divided by the apparent stoichiometric solubility product for aragonite, the polymorph of CaCO$_3$ produced by corals. Even with increasing ocean acidification, tropical surface waters are expected to remain supersaturated with respect to aragonite (Ωarag > 1) (Kleypas *et al*., 1999; Caldeira & Wickett, 2003; Hoegh-Guldberg *et al*., 2007; Zeebe *et al*., 2008). A number of studies have shown that coral calcification rates are directly related to seawater Ωarag, following from the geochemical model whereby the abiotic CaCO$_3$ precipitation rate from seawater is a function of saturation state (Mucci, 1983). Other studies have reported a
strong negative correlation between pCO₂ enrichment, and/or seawater acidification with hydrochloric acid, both of which reduce Ωarag, and a decreased rate of calcification in diverse marine calcifiers including coccolithophorids (Riebesell et al., 2000; Zondervan et al., 2001, 2002; Sciandra et al., 2003; but see Iglesias-Rodríguez et al., 2008 for an opposite effect), foraminiferans (Spero et al., 1997; Bijma et al., 1999, 2002), coralline red algae (Agegian, 1985; Kuffner et al., 2008), coral reef assemblages (Broecker & Takahashi, 1966; Ohde & van Woesik, 1999; Langdon et al., 2000; Leclercq et al., 2000, 2002), adult scleractinian corals (Gattuso et al., 1998; Marubini & Atkinson, 1999; Marubini & Thake, 1999; Marubini et al., 2001, 2003, 2008; Marshall & Clode, 2002; Reynaud et al., 2003; Langdon & Atkinson, 2005; Renegar & Riegl, 2005; Schneider & Erez, 2006; Anthony et al., 2008; Jokiel et al., 2008) and coral larvae (Albright et al., 2008; Cohen et al., 2009). The consensus estimate based on the relationship between calcification and Ωarag, is that the rate of calcification in scleractinian corals will decrease 17–37% as a result of reduced seawater [CO₃²⁻] due to a doubling of preindustrial levels of atmospheric CO₂ by the end of this century (Gattuso et al., 1999; Kleypas et al., 1999). While the studies above show drastic reductions in coral calcification as response to ocean acidification, there are indications that such responses are not ubiquitous. Although their data set does not include calcification rates or growth under normal seawater conditions, Atkinson et al. (1995) have reported long-term high growth rates for 57 species of coral growing in seawater with very low pH (~7.6), low [CO₃²⁻] (~65 μM) and low Ωarag (~1), but slightly elevated [HCO₃⁻] (~2000 μM), which indicate that corals can continue to calcify under extremely low [CO₃²⁻]. In addition, Ries et al. (2008) and Rodolfo-Metalpa et al. (2009) have reported that some temperate coral species showed no response to CO₂ enrichment.

A predominant role for seawater CO₃²⁻ in coral calcification, either by passive or active uptake, is not consistent withmorphological or physiological studies. If passive uptake were important, and corals did not otherwise regulate internal cytoplasmic and internal fluid chemistries, a reduction of seawater [CO₃²⁻] could be expected to reduce Ωarag at the site of calcification, decreasing calcification. However, the calicoblastic epithelium, which separates the site of calcification from external seawater in corals, is characterized by septate junctions between cells, creating a tight epithelium and preventing significant ion diffusion between seawater and the calcifying fluid (Tambutte et al., 2007). While ⁴⁰Ca²⁺ and ¹³C tracers are able to permeate the oral tissues of corals through passive diffusion, active transport is necessary to move them to the site of calcification (Furla et al., 2000). Since passive CO₃²⁻ transport is unlikely, a CO₃²⁻ transport system would be necessary for reduced seawater [CO₃²⁻] to directly reduce calcification, but one has never been identified in corals (Langdon & Atkinson, 2005) although it has been sought (Al-Moghrabi et al., 1996; Goiran et al., 1996). In contrast, HCO₃⁻ transporters and channels have been identified and physiological models indicate that HCO₃⁻ is the species transported to the calcification site and thus the major carbon source for calcification (Furla et al., 2000). Furthermore, several studies have proposed that CO₂, much of which may be respiratory, is the predominant carbon species that is transported or diffuses into the calcifying space for skeletal growth in corals (reviewed in Cohen & McConnaughey, 2003). In either case, it seems unlikely that a direct supply of CO₂ from ambient seawater is the major carbon source for calcification. This presents a major challenge in rectifying the negative effects of ocean acidification shown experimentally and our understanding of bio-mineralization in corals.

Ocean acidification also results in increased [HCO₃⁻], although the role of an increase in [HCO₃⁻] on biological calcification has not been generally considered in ocean acidification studies. Previous studies have indicated a positive relationship between [HCO₃⁻] and coral calcification rates. Marubini & Thake (1999) found Porites porites had higher calcification rates in treatments in which [HCO₃⁻] were enriched compared with ambient. Similarly, at constant pH, [HCO₃⁻] enrichment stimulated calcification in Acropora eurystoma (Schneider & Erez, 2006), and stimulated both calcification and photosynthesis in Acropora sp., P. porites (Herfort et al., 2008) and Stylophora pistillata (Marubini et al., 2008). While these studies indicate a positive influence of [HCO₃⁻] on calcification rates, the treatments used were such that [CO₂] increased in proportion to the [HCO₃⁻] increase, and thus the roles CO₂ vs. HCO₃ could not be distinguished. If seawater [HCO₃⁻] plays a role in calcification in corals, one might expect a positive correlation with the shift in equilibrium concentrations of CO₂ to HCO₃ due to seawater acidification, but this has not been demonstrated. This indicates that any positive benefit of increased HCO₃ might be offset by some other negative influence of acidification.

Reduced seawater pH (increased [H⁺]) may itself affect coral calcification. Precipitation of CaCO₃ results in the production of H⁺ at the site of calcification, which must be removed for further calcification to occur (Zoccola et al., 2004). Protons are removed from the site of calcification through the action of a Ca²⁺ / H⁺-ATPase present in the calicoblastic epithelium, which also delivers the required Ca²⁺ (Zoccola et al., 2004). Ultimately these H⁺ must be expelled from the
calicoblastic cells and into seawater to prevent tissue acidosis, although the details of this pathway are unknown. Cohen & McConnaughey (2003) argue, based on several lines of evidence, that the protons produced during calcification are neutralized by the conversion of HCO$_3^\text{-}$ to CO$_2$ which can then be taken up by the zooxanthellae or diffuse back to the calcifying space. In this scenario, the Ca$^{2+}$ is important for the removal of H$^+$ from the calcifying space. Depending on the mechanism of H$^+$ transport away from the calicoblastic epithelium, reduced seawater pH might impede H$^+$ removal from the site of calcification, and possibly Ca$^{2+}$ transport, or indirectly result in tissue acidosis. However, direct effects of reduced pH on cellular function have only rarely been invoked as possible causes for reduced coral calcification rates due to ocean acidification (Gattuso et al., 1999; Langdon & Atkinson, 2005).

Previous studies have examined the effects of varying CO$_3^{2-}$, HCO$_3^-$ and pH in various permutations, but few studies have attempted to use chemical manipulations that allow each parameter to be discriminated from the others. Schneider & Erez (2006) concluded that CO$_3^{2-}$ was the dominant control of calcification even though their data showed similar relationships between calcification and pH, DIC and TA, while the relationship with HCO$_3^-$ was not considered. Marubini et al. (2008) used three levels of pH at ambient (2 mM) and enriched (4 mM) HCO$_3^-$. They reported a positive relationship between calcification and CO$_3^{2-}$ as well as clear stimulation of calcification by HCO$_3^-$ enrichment, but the absence of low HCO$_3^-$ concentrations in their experiments did not allow for a full examination of the calcification–HCO$_3^-$ relationship. Herfort et al. (2008) used a wide range of HCO$_3^-$ concentrations and demonstrated a strong relationship between HCO$_3^-$ and calcification, but HCO$_3^-$ and CO$_3^{2-}$ covaried in their experiment such that the two could not be considered separately. Thus, there is a large body of data that indicates a negative effect of ocean acidification on coral calcification, but because of the covariance of carbonate parameters the assignment of cause to only one of the carbonate parameters is not unambiguous.

The objective of our study was to determine how variations in [HCO$_3^-$], [CO$_3^{2-}$] and pH affect coral calcification rates, and to discriminate among the effects of the three. This was accomplished with a series of incubations in carefully manipulated seawater chemistries that allowed each of these three parameters to be investigated. In particular, the effects of pH and [CO$_3^{2-}$] were separated by holding each parameter constant and

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**Table 1** Summary of the target levels of pH$_T$ and [CO$_3^{2-}$] used in the experimental treatments, and of the resulting differences in calcification rates (% change compared with control/control × 100; means ± SE)

| CO$_3^{2-}$ μmol kg$^{-1}$ | pH$_T$ |
|-----------------------------|--------|
|                            | (Normal) | (Low) | (Very low) |
|                            | 8.06     | 7.78  | 7.60       |
|                            |          |       |            |
| (Normal) 260 μmol kg$^{-1}$ |          |       |            |
|                            | (Control chemistry) |       |            |
| HCO$_3^-$ ~ 1800 μmol kg$^{-1}$ |       |       |            |
| pCO$_2$ ~ 390 μatm         |       |       |            |
| TA ~ 2470 μeq kg$^{-1}$   |       |       |            |
| Calcification = 100 ± 3.3%|       |       |            |
| nd                         |       |       |            |
| (Low) 150 μmol kg$^{-1}$   |          |       |            |
|                            | (Low pH, low CO$_3^{2-}$) |       |            |
| HCO$_3^-$ ~ 1060 μmol kg$^{-1}$ |       |       |            |
| pCO$_2$ ~ 230 μatm        |       |       |            |
| TA ~ 1515 μeq kg$^{-1}$  |       |       |            |
| Calcification = 57 ± 1.3%|       |       |            |
| nd                         |       |       |            |
| (Very low) 105 μmol kg$^{-1}$ |          |       |            |
|                            | (Normal pH, very low CO$_3^{2-}$) |       |            |
| HCO$_3^-$ ~ 740 μmol kg$^{-1}$ |       |       |            |
| pCO$_2$ ~ 170 μatm        |       |       |            |
| TA ~ 1100 μeq kg$^{-1}$  |       |       |            |
| Calcification = 46 ± 1.1%|       |       |            |
| nd                         |       |       |            |

Treatments are designated by pH and [CO$_3^{2-}$] relative to natural seawater, but specific changes in [HCO$_3^-$] were also targeted. The [HCO$_3^-$], pCO$_2$ and total alkalinity (TA) are included for each treatment. nd, no data (not used). For clarity, variance estimates for the carbonate parameters are not included here but are included in Table 2. The three treatments along the diagonal (pale grey) directly mimic ocean acidification by CO$_2$. The other three treatments used manipulations that forced variations of the parameters that are not normally encountered in seawater in order to investigate the role of each more independently.

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manipulating the other to fall in the range of present and future values due to ocean acidification (Table 1). Bicarbonate concentration was manipulated over a wide range and in ways that allowed separation of its covariance from pH and [CO$_3^{2-}$]. The experimental coral chosen for this study is the ecologically important Caribbean species, *M. auretenra*, ("yellow pencil coral", commonly misidentified as *M. mirabilis*; Locke *et al*., 2007) which forms large, dense clusters of thin branches that offer habitat for a large variety of reef microfauna and flora. It is particularly abundant in the southern Caribbean and can constitute more than one-third of the live flora. It is particularly abundant in the southern Caribbean and can constitute more than one-third of the live flora.

**Materials and methods**

*Collection, transport and maintenance of M. auretenra*

Clusters of *M. auretenra* branches were collected at 9 m depth from the fringing reef off of the Curacao Seaquarium, Netherlands Antilles on February 21, 2007. Branches approximately 3–5 cm in length were cut from the clusters and thin pieces of acrylic rod 2 cm in length were glued into holes drilled into the branch bases using cyanoacrylate gel to provide a handle with which to manipulate the branches. Any exposed skeleton was sealed with cyanoacrylate gel to seal off the skeleton from external seawater. All preparations described above were completed within 48 h of collection. The branches were kept in a flowing seawater system with shade cloth attenuated natural sunlight (by 50%) to recover from handling. They were transported to the University of North Carolina Wilmington (UNCW) on April 12, 2007.

At UNCW, the corals were housed in a large 1000 L recirculating seawater system located at the Center for Marine Science. The system was home to a variety of corals, sea urchins, snails, crustose coralline algae and small numbers of other invertebrates. A number of submersible pumps provide extra circulation and turbulence within the system. The seawater was maintained at a salinity of ca. 36, temperature of 27–28°C, total alkalinity (TA) = 2400–2550 μmol kg$^{-1}$ and total-scale pH$_T$ = 8.0–8.1. Figure 1 shows the history of the TA concentrations within the recirculating system during the time frame of the experimental incubations, as well as the TA values of the Dickson seawater standard run as an unknown. Nutrients were measured periodically and were typically low: nitrate <1.0 μmol kg$^{-1}$, phosphate <0.2 μmol kg$^{-1}$. Regular partial water exchanges were performed with 1 μm filtered and UV-treated natural seawater obtained from the intracoastal waterway adjacent to the Center for Marine Science. A saturated solution of Ca(OH)$_2$ was dripped into the system to replace calcium and alkalinity lost to calcification by corals and sea urchins in the seawater system and to the protein skimmer (Sondervan, 2001). Light was provided by metal halide lamps at an irradiance of ca. 200 μmol photons m$^{-2}$ s$^{-1}$ on a 12 h light: dark cycle controlled by a timer.

The corals were fed twice weekly with newly hatched *Artemia* nauplii; however, feeding was discontinued 3 days before any incubation so that there would be no contribution of digestion to the metabolic rates of the experimental corals (Szmant-Froelich & Pilson, 1984). Feeding was resumed in between incubations as long as the interval was longer than 3 days.

![Fig. 1 Total alkalinity (TA) in the recirculating seawater system in which the corals were maintained, plotted against date (= dark symbols). TA varied from ~2400 to 2550 μEq kg$^{-1}$ during these experiments, but did not show a significant trend over time (P > 0.05). Also shown are the mean values (n = 3) obtained for the titrations of the Dickson standard (= open symbols) when run as unknowns. Symbols are mean ± SD from three titrations each day; the error bars are smaller than the size of the symbols. Over the course of the experiments, the Dickson standard was analyzed 48 times with a range of 2198–2205 μmol kg$^{-1}$; mean ± SD 2202 ± 2.0 μmol kg$^{-1}$, which compared well with the certified value of 2201.20 ± 0.66 μmol kg$^{-1}$ TA.](image)
Calcification rates were measured with short laboratory incubations using the TA anomaly method because it is nondestructive and can be used to take repeated measurements using the same individual corals incubated under different experimental conditions (Chisholm & Gattuso, 1991). Thus, TA by necessity decreased during the incubations but the decreases were only ca. 1.5–6.5% of the initial concentration, a small difference in comparison with the differences among treatments. This method relies on the stoichiometric relationship wherein two equivalents of alkalinity are removed per mole of carbonate mineral precipitated, described by

$$\text{Ca}^{2+} + 2\text{HCO}_3^- \rightarrow \text{CaCO}_3 + \text{CO}_2 + \text{H}_2\text{O}.$$  

Calcification rates were calculated from the changes in TA during the incubations, corrected for any change in TA in the seawater-only control beakers, and multiplied by the mass of seawater in which the corals were incubated as described by the equation

$$\text{Calcification} = [(\text{TA} \times 2) \times \text{Mass}_{\text{sw}}] / \text{Time},$$

with units of $\mu$mol CaCO$_3$ h$^{-1}$. Rates were normalized to the surface area of the growing branch tips. Each coral sample consisted of two small branches, each ca. 3 cm long, so as to obtain sufficient coral biomass to effect a change in alkalinity of $\sim 100\mu$Eq kg$^{-1}$ in the beakers during the short 2 h incubation period.

**Preparation of seawater chemistries**

Six different water chemistries were prepared for this study: (1) Normal pH and CO$_3^-$ (Control chemistry), (2) Normal pH, Low CO$_3^-$, (3) Normal pH, Very low CO$_3^-$, (4) Low pH, Normal CO$_3^-$, (5) Low pH, Low CO$_3^-$ and (6) Very low pH, Very low CO$_3^-$ (Table 1). These chemistries were produced by manipulating TA and DIC to arrive at the desired values. TA and pH$_T$ were verified using spectrophotometric methods before each incubation.

In order to produce each chemistry, 15 L of water from the recirculating seawater system was placed in a 20 L plastic tub with a small pump to provide rapid circulation. For the Control chemistry, if necessary pH$_T$ was adjusted to $\sim 8.06$ by bubbling CO$_2$ to lower pH slightly. To produce Low pH, Low CO$_3^-$ and Very low pH, Very low CO$_3^-$, CO$_2$ was bubbled into well-circulated seawater until the desired pH was obtained (pH$_T$ $\sim 7.78$ and 7.60, respectively). For Normal pH, Low CO$_3^-$ and Normal pH, Very low CO$_3^-$, seawater was first acidified with 2N HCl to pH$_T$ < 6.5 and then aerated for 60 min to drive off CO$_2$. The TA were then adjusted to 1500 $\mu$Eq kg$^{-1}$ (Normal pH, Low CO$_3^-$ or 1100 $\mu$Eq kg$^{-1}$ (Normal pH, Very low CO$_3^-$) by adding 1.0 N NaOH slowly to an area of strong circulation so as to prevent the precipitation of hydroxides. CO$_2$ was simultaneously bubbled into this seawater and pH monitored with a pH meter to ensure that pH did not rise above 8.6 during the addition of NaOH. Last, CO$_2$(g) was bubbled until the desired pH was obtained ($\sim 8.06$). For Low pH, Normal CO$_3^-$, it was necessary to increase both TA and DIC in order to achieve normal [CO$_3^-$] at low pH. This was done by adding $\sim 2$ g NaHCO$_3$ to 15 L of seawater which raised the TA to $\sim 4150$ $\mu$Eq kg$^{-1}$; CO$_2$ was then bubbled into the seawater until the desired pH was obtained ($\sim 7.78$). For altered chemistries, adjustments to TA were made and verified before adjustments to pH. Table 2 summarizes the mean seawater chemistries at the beginning of the incubations.

**Incubation procedures**

Six 250 mL Pyrex beakers were used for the incubations: three contained pairs of branches of M. auretenra, and three contained seawater only (with the same chemistry used with the corals) to correct for changes in seawater.

**Table 2 Average ± SD initial seawater chemistry conditions for each treatment**

| Treatment                        | pH$_T$  | TA    | pCO$_2$ | DIC     | CO$_3^-$ | HCO$_3^-$ | CO$_2^-$ | O$_{arag}$ |
|----------------------------------|---------|-------|---------|---------|----------|-----------|----------|-----------|
| Control chemistry (as control)   | 8.07 ± 0.01 | 2469 ± 29 | 393 ± 12 | 2101 ± 28 | 10.3 ± 0.3 | 1827 ± 26 | 264 ± 4 | 4.22 ± 0.07 |
| Control chemistry (as treatment) | 8.07 ± 0.01 | 2462 ± 23 | 391 ± 11 | 2094 ± 24 | 10.2 ± 0.3 | 1820 ± 23 | 264 ± 3 | 4.22 ± 0.05 |
| Normal pH, low CO$_3^-$          | 8.07 ± 0.01 | 1515 ± 37 | 235 ± 10 | 1250 ± 33 | 6.14 ± 0.25 | 1088 ± 29 | 157 ± 5 | 2.51 ± 0.08 |
| Normal pH, very low CO$_3^-$     | 8.06 ± 0.01 | 1110 ± 18 | 171 ± 3  | 891 ± 12  | 4.46 ± 0.07 | 777 ± 8  | 110 ± 4 | 1.76 ± 0.06 |
| Low pH, normal CO$_3^-$          | 7.79 ± 0.01 | 4184 ± 59 | 1480 ± 31 | 3887 ± 56 | 38.7 ± 0.8 | 3579 ± 52 | 269 ± 5 | 4.30 ± 0.07 |
| Low pH, low CO$_3^-$             | 7.78 ± 0.01 | 2471 ± 30 | 876 ± 37 | 2273 ± 33 | 22.9 ± 1.0 | 2094 ± 33 | 156 ± 3 | 2.49 ± 0.04 |
| Very low pH, very low CO$_3^-$   | 7.60 ± 0.01 | 2485 ± 10 | 1406 ± 45 | 2367 ± 15 | 36.8 ± 1.2 | 2221 ± 15 | 109 ± 2 | 1.74 ± 0.03 |

TA reported as $\mu$mol kg$^{-1}$, pCO$_2$ as atm, and CO$_2$, DIC, HCO$_3^-$, and CO$_2^-$ as $\mu$mol kg$^{-1}$. Spectrophotometrically measured TA and pH were used to calculate the other parameter with CO2SYS (Lewis & Wallace, 1998). The water used for all incubations was taken from the recirculating seawater system in which the corals were maintained (= Control chemistry: Normal pH, Normal CO$_3^-$).
chemistry during the incubations not caused by the corals. Coral branches were secured within the incubation chambers on stands constructed from PVC and plastic mesh. Water stirring was provided in each beaker with a magnetic stir bar and was sufficient to homogenize an aliquot of dye within ~10 s. The beakers were incubated within a temperature controlled water bath sitting atop a 15 place Ika® stir table. Light during the incubations was provided by a 250 W metal halide lamp at an intensity of 110 ± 10 μmol photons m⁻² s⁻¹. Temperature was maintained at 28.0 ± 0.3 °C with a submersible aquarium heater and the occasional addition of an icepack to the temperature bath.

Incubation chambers were left open to the atmosphere to allow pH to be monitored with a pH meter. pH was closely regulated and not allowed to change from the target levels throughout the incubations. When needed, CO₂ was bubbled into the individual beakers while monitoring with the pH electrode to maintain pH and make up for CO₂ lost to photosynthesis or to the atmosphere. Because of this time-consuming need to constantly check and adjust pH, only six beakers could be incubated at a time.

Two incubations were performed per day: one was a ‘treatment’ incubation with one of the five altered seawater chemistries, and one was a control incubation with normal seawater. Each treatment chemistry was performed twice: once with the control incubation first in the day and the treatment incubation second, and once with the treatment incubation first in the day and the control incubation second. The order in which the treatment incubations were conducted was randomized. Twice per incubation series, both the first and second incubations were performed in the control chemistry with one assigned as a treatment incubation and the other assigned as a control incubation. This allowed us to compare each individual coral’s performance in the altered chemistries to its performance in the control chemistry within the same day. The methodology ensured that measured responses in calcification were induced by the chemistries investigated and were not an artifact of experimental manipulation or confounded by normalization to surface area. The entire experiment series was performed twice, each time with three different corals (Supporting Table S1).

To begin each daily incubation set, the corals were transferred from the recirculating seawater system to their beakers which were submerged within the 20 L acclimation bath containing the seawater chemistry being investigated. The corals were allowed 3 h to acclimate before an incubation was begun. Previous work has shown that coral calcification adjusts to new chemistry conditions in <3 h (Furla et al., 2000; Langdon & Atkinson, 2005). Mixing sufficient to cause swaying in the coral polyps was provided by a submersible pump. The large volume of water (15 L) compared with the tissue of the three corals in the acclimation bath ensured that the water chemistry remained stable during this adjustment period. pH was monitored frequently and maintained with CO₂ additions as needed. After the 3 h acclimation period, samples for initial TA and pH were taken from the acclimation bath. Each incubation beaker was then carefully removed from the acclimation bath and placed directly into the temperature-controlled water bath on the Ika® (IKA Works, Wilmington, NC, USA) stir table.

Corals were incubated for 2 h, maintaining pH within each beaker with CO₂ additions as described above. Temperature of the water bath and pH of each chamber were measured and recorded every 20 min. At the end of an incubation, the seawater in each coral beaker was emptied into four preweighed 50 mL centrifuge tubes: three for TA determination, one for final spectrophotometric pH determination. Each water-filled tube was then weighed and summed to determine the total mass of seawater in each chamber (~200 g). Final TA and pH samples were also taken from each of the three seawater-only control beakers, the results of which were averaged and used to correct for nonbiologically related changes in chemistry.

At the end of the first incubation, the beakers with branches were transferred back to the acclimation bath filled with the second water chemistry of the day, and given 3 h for acclimation. A second incubation for the day was then performed in this new water chemistry, as described above. After this second incubation all coral branches were returned to the recirculating seawater system.

**Analytical procedures**

Seawater samples for pH measurements were collected and stored in sealed 50 mL centrifuge tubes and run within 1 h of collection. pHT was determined spectrophotometrically using the pH indicator m-cresol purple and an Ocean Optics spectrophotometer in conjunction with the equations of Clayton & Byrne (1992) at in situ conditions of S = 36 and T = 28°C. This method yielded a precision of ca. ±0.004 pH units in triplicate subsamples from the seawater system examined before experimentation. Therefore, all pH values reported in tables and figures and those used for calculations with CO2SYS (Lewis & Wallace, 1998) were those obtained spectrophotometrically. During incubations, pH was also measured with an Orion pH meter (model 720A, Orion Research, Boston, MA, USA) and a ROSS Ultra combination electrode calibrated with NBS buffers (precision of ca. ±0.005 pH units) to monitor relative
changes in pH throughout the incubations. The pH meter (with accounting for the offset between the NBS and total pH scales) was used simply to verify that the pH was maintained at the initial pH ± 0.02 during incubations, as described above.

Seawater samples for TA measurements were also stored in sealed 50 mL centrifuge tubes and analyzed in triplicate within 48 h of sampling. TA was measured using the method and equations of Yao & Byrne (1998) using ~ 0.1 N HCl standardized against calibrated seawater standard Batch 81 (TA = 2101.20 ± 0.66 μmol kg⁻¹) provided by Dickson ( Scripps Institute of Oceanography, La Jolla, CA, USA). A seawater sample of ca. 30 g was weighed to ± 1 mg. Sufficient acid, to reduce the pH of the sample to ~ 4.0, was pipetted into the sample and the sample reweighed to ± 1 mg. The acidified sample was aerated with N₂ for 5 min to drive off excess CO₂ and the pH indicator bromocresol green added. The absorbance of the sample was then read using an Ocean Optics spectrophotometer. In-cell temperature was measured with a mercury thermometer. This method yielded a precision of ca. ± 2 μEq kg⁻¹ in triplicate seawater samples. Fresh Dickson certified seawater TA standards were run frequently as unknowns (in triplicate) to ensure the accuracy of TA determinations (Fig. 1).

The aragonite saturation state and other CO₂ chemistry parameters were calculated using the program CO2SYS (Lewis & Wallace, 1998) given measured values of pH, TA, salinity and temperature. Pressure effects as well as orthophosphate and silicate concentrations were assumed to be negligible. Salinity was determined to within ± 0.5 with a hand-held refractometer and temperature was measured with a mercury thermometer accurate to ± 0.05 ºC.

Normalization of calcification rates

Calcification rates were calculated two ways. One way was to normalize the changes in TA to the surface areas of the calcifying tips of the experimental corals measured using the aluminum foil method (Marsh, 1970) and the second was to compare the calcification of each coral in the treatment chemistry against itself on the same day in the control chemistry. Our experimental design of conducting both a control and treatment incubation each day was chosen to allow this second method of data analysis.

Examination of skeletons of M. auretenra that had been stained with alizarin red-s and allowed to calcify for several weeks showed that only the distal 5 ± 1 mm of branches were active in calcification. Rinkevich & Loya (1984) reported that another similar branching coral, S. pistillata, also calcifies on the tips vs. further down the branch. Therefore, only the area of the distal 5 mm of each of the tips within the sample (some branches had two or more tips) was measured. Tip surface areas of the three corals used in the first incubation Series were 13.56, 13.12 and 12.25 cm² for corals 1, 2 and 3, respectively, while those in Series 2 were 10.83, 10.18 and 8.55 cm² for corals 4, 5 and 6, respectively. Calcification rates were expressed as nmol CaCO₃ cm⁻² h⁻¹ (Supporting Table S1 and Fig. 2a and b).

The second method is similar to that used in a number of studies (e.g. Ferrier-Pagès et al., 2000; Reynaud et al., 2003; Marubini et al., 2008) in which coral
physiological rates are normalized against their individual initial weight or condition. This method avoids issues related to variation in individual size and functions among samples, and is reported as a percent change.

Calcification as % same day control
$$= \left[ \frac{\text{Treatment rate}}{\text{Control rate}} \right] \times 100.$$  

Statistical analyses

To determine how area normalized calcification rates in the treatments compared with calcification rates under control conditions, a Kruskal–Wallis one way ANOVA on ranks followed by a Dunn’s multiple comparison of each treatment against the control was performed using SIGMASTAT 3.5. For the comparison among treatment using the percent of control, the data were first log arcsine transformed before analysis by ANOVA, and a Bonferroni’s test for differences among means was used as a post-hoc test with BIOSTAT, 2007. Regressions in Fig. 3 were fit with SIGMAPLOT 9.0.

Results

Absolute rates of calcification

Surface area normalized rates of calcification of the six sets of *M. auretenra* varied from 296 ± 49 to 467 ± 87 nmol CaCO$_3$ cm$^{-2}$ h$^{-1}$ (mean ± SD for each coral; Fig. 2a). Per area rates of calcification reported in other studies have been similarly variable (Gattuso et al., 1998; Langdon & Atkinson, 2005). A one-way ANOVA indicated significant differences among individual corals ($P < 0.05$), and a Bonferroni post-hoc test yielded two groups among the six corals. The three corals in Series 1 calcified at statistically similar rates under control chemistry conditions, and at significantly higher rates than two of the corals in Series 2 (Fig. 2a).

Fig. 3 Plots of mean (± SE) calcification rates of *M. auretenra* against concentrations of the various DIC parameters that resulted from the seawater treatments used in this study. Calcification rates are plotted against the mean of the initial and final pH values and [CO$_3^{2-}$] and [HCO$_3^-$]; horizontal error bars indicate the ranges of the initial and final values and for most cases are smaller than the sizes of the symbols. Calcification rates of each coral in each experimental treatment expressed as a percent of the calcification rate of the same coral measured on the same day under control seawater conditions. The ‘Control’ values are for corals that were incubated under control conditions both times during that day. (a) Calcification rates plotted against pH. (b) Calcification rates plotted against [CO$_3^{2-}$] (lower x axis) and ᵢارة (upper x axis). Upper regression includes treatments at constant total alkalinity (TA) and elevated pCO$_2$. Lower regression includes treatments at constant pH and reduced DIC/TA. Square data point in upper right is elevated [HCO$_3^-$], normal [CO$_3^{2-}$], and reduced pH. (c) Calcification rates plotted against [HCO$_3^-$]. Where error bars are not evident, it is because the SE was smaller than the size of the symbol.

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To examine whether absolute rates of calcification for *M. auretenra* measured in our laboratory system under control conditions were within the normal range for this species, rates of calcification obtained in the control incubations were compared with rates measured in field experiments in Curacao taking into consideration that our measurements were intentionally conducted at low light levels of only 110 μmol photons m\(^{-2}\) s\(^{-1}\). This comparison was calculated as growth rate = hourly rate of calcification (average of 400 nmol cm\(^{-2}\) h\(^{-1}\)) × 12 h of calcification per day (thus an underestimate of expected 24 h calcification) × surface area of experimental coral (average of 11.4 cm\(^2\) × growth period to match the field growth experiment. These calculations yielded growth rates of 2.0 g yr\(^{-1}\) compared with 1–4 g yr\(^{-1}\) in Jamaïca (Leichter & Genovese, 2006). In addition, 35 branches of *M. auretenra* grown for 20 days in the flowing seawater of the Curacao Sea Aquarium under attenuated sunlight (ca. 500 μEin m\(^{-2}\) s\(^{-1}\) in March 2009 grew at the average rate of 692 nmol cm\(^{-2}\) h\(^{-1}\) if net skeletal weight gain was assumed to be secreted during 12 light hours per day (A. M. Szment and R. F. Whitehead, unpublished data). Thus, the corals used in the present study calcified at a rate similar to those in the field and in field laboratory experiments. In addition, linear regressions of the control calcification rates for each coral showed no significant trends in calcification over the entire time frame of the incubations (August 2007 to January 2008) for any of the corals (*P* > 0.05; data not shown).

**Effects of chemistries on calcification rates**

Mean calcification rates of *M. auretenra* normalized to surface area for each of the seawater chemistries tested are presented in Fig. 2b. A Kruskal–Wallis one way ANOVA on ranks was highly significant (*P* < 0.001) and the Dunn’s post-hoc test showed that the only two treatments that differed from control rates were the [Normal pH, Low CO\(_3^{2-}\)] and the [Normal pH, Very Low CO\(_3^{2-}\)] (Fig. 2b), both of which also had [HCO\(_3^{-}\)] ≤ 1000 μM. In contrast, the three treatments with low pH were not statistically different from controls. These low pH treatments were intentionally manipulated to have [HCO\(_3^{-}\)] higher than normal seawater.

Comparisons among treatments using calcification rates calculated as the percent of same day control rate were best for showing the magnitude of the chemistry on calcification rates, and yielded additional statistically significant differences. As in the above analysis, rates of calcification in the Normal pH, Low CO\(_3^{2-}\) treatment and the Normal pH, Very Low CO\(_3^{2-}\) treatment were 43% and 54% lower than in the control chemistry, respectively (*P* < 0.000 in both cases); [HCO\(_3^{-}\)] was low in both of these chemistries. The Low pH, Normal CO\(_3^{2-}\) treatment ([HCO\(_3^{-}\)] of >2000 μM) resulted in a 21% increase in calcification rate relative to the control chemistry (*P* = 0.001). Rates of calcification in the Low pH, Low CO\(_3^{2-}\) and Very Low pH, Very Low CO\(_3^{2-}\) treatments were not significantly different from the control (*P* > 0.05); [HCO\(_3^{-}\)] were normal in both of these chemistries.

When calcification rates were plotted against pH, there was no clear pattern among the treatments, suggesting that pH alone was not a major factor in determining the rate of calcification in *M. auretenra* (Fig. 3a). In fact, high rates of calcification were obtained over the entire pH range tested. The only treatments that resulted in reduced calcification were two at high pH.

When calcification rates were plotted against [CO\(_3^{2-}\)], there were three different patterns of response, depending on the type of seawater manipulation (Fig. 3b). In the first response pattern (top regression line Fig. 3b), which included incubations at constant TA but increased pCO\(_2\) (equivalent to the real-world CO\(_2\) enrichment scenario), calcification rates did not change significantly. Both [CO\(_3^{2-}\)] and pH were reduced in these incubations but [HCO\(_3^{-}\)] was elevated (14–20% depending on treatment). A linear regression of calcification rate vs. [CO\(_3^{2-}\)] yielded *r*\(^2\) = 0.02 and a slope that was not significantly different from zero (*P* > 0.05). In the second response pattern (lower regression line Fig. 3b), which included treatments with constant pH but reduced TA, rates of calcification decreased significantly with reduced [CO\(_3^{2-}\)]. In these incubations, both [CO\(_3^{2-}\)] and [HCO\(_3^{-}\)] were proportionally lower while pH was constant. A linear regression of calcification rate vs. [CO\(_3^{2-}\)] yielded *r*\(^2\) = 0.88 and a slope that was highly significant (*P* < 0.000). In the third response (square data point, upper right in Fig. 3b) in which [CO\(_3^{2-}\)] was maintained at the same level as the control but [HCO\(_3^{-}\)] was higher and pH lower, significantly higher rates of calcification were obtained (*P* < 0.001). Thus, the observed patterns of calcification rate in *M. auretenra* were not clearly attributable to changes in [CO\(_3^{2-}\)], or thus Ωarag. At low [CO\(_3^{2-}\)], calcification rates were low only if [HCO\(_3^{-}\)] was also low, but at the same low levels of [CO\(_3^{2-}\)] calcification rates were high when [HCO\(_3^{-}\)] was high.

When calcification rates were plotted against [HCO\(_3^{-}\)], there was a clear trend toward higher calcification rates with higher [HCO\(_3^{-}\)] over the entire range of study (Fig. 3c). An exponential regression of the form \(y = y_0 + a(1 - e^{-bx})\), chosen based on the apparent saturable kinetic form (Gattuso et al., 1998), yielded an *r*\(^2\) = 0.78, which was highly significant (*P* < 0.000). These results taken together demonstrate that *M. auretenra* responded strongly to changes in [HCO\(_3^{-}\)] and showed very little if any response to changes in [CO\(_3^{2-}\)], Ωarag or pH.
Discussion
There is little doubt that coral calcification responds to changes in seawater chemistry: the question is which species of the carbonate system or pH itself are most responsible for the observed responses. The manipulations of seawater chemistry performed in this study allow each of the carbonate parameters to be examined separately, and show that the relationship between calcification by M. auretenra and [HCO$_3^-$] was the most consistent among the variables considered. The form of the relationship between [HCO$_3^-$] and calcification rate in M. auretenra suggests saturable kinetics, as would be expected for the HCO$_3^-$ transporter implicated in coral calcification (reviewed by Gattuso et al., 1999; Furla et al., 2000). At constant pH, calcification rates decreased significantly when both [HCO$_3^-$] and [CO$_3^{2-}$] decreased, but calcification rates did not decrease when [CO$_3^{2-}$] fell over the same range when [HCO$_3^-$] was kept high. Thus, significantly different rates of calcification were obtained at the same levels of [CO$_3^{2-}$] and Ωarag due to variation in [HCO$_3^-$], indicating that [HCO$_3^-$] drove this response. The response of M. auretenra is consistent with the generally accepted physiological model of calcification using HCO$_3^-$ as the primary skeletal carbon source, but is at odds with a geochemically based aragonite saturation state model of coral calcification response to ocean acidification.

Langdon & Atkinson (2005) compiled the results of studies that examined the effects of altered Ωarag on coral calcification and found that the responses fell into two general groups: a ‘low sensitivity’ group where doubled CO$_2$ resulted in a 0–18% reduction in calcification rates, and a ‘high sensitivity’ group where doubled CO$_2$ resulted in a 40–83% reduction in calcification rates. The data from the current study indicates that M. auretenra would fall in the high sensitivity group when both [CO$_3^{2-}$] and [HCO$_3^-$] were low, but in the low sensitivity group whenever [HCO$_3^-$] are near normal seawater concentrations or higher.

Insensitivity to CO$_2$ acidification by some corals is supported by both observational and experimental studies. Pelejero et al. (2005) examined skeletal cores of coral that had naturally been exposed to a range of pH values similar to those expected this century due to ocean acidification. Over the 300 years record, mean pH as determined by skeletal Boron isotopic data varied from ~8.2 to 7.9 (5-year resolution) with a ~50 year periodicity that was strongly correlated with the Inter-decadal Pacific Oscillation. Assuming constant TA of 2300 µEq kg$^{-1}$ (present-day value), the pH variation would be accompanied by a pCO$_2$ range of 250–590 µatm and Ωarag range of ~3–4.5. Despite CO$_2$ acidification over this range, neither calcification nor linear extension showed any trend over the 300-year period (~6 high- to low-pH cycles). Reynaud et al. (2003) found that S. pistillata was insensitive to doubled CO$_2$ at 25°C, but experienced a 50% reduction in calcification rate at a higher temperature of 28°C. Oppositely, Anthony et al. (2008) found that doubled CO$_2$ had no effect on calcification in Acropora intermedia and caused a ~19% increase in calcification in Porites lobata at a temperature of 28–29°C, but the same acidification decreased calcification by ~19% and 12% in A. intermedia and P. lobata, respectively, at a lower temperature of 25–26°C. Thus, some corals shift between insensitivity and high sensitivity to CO$_2$ acidification based on changes in temperature. Likewise, Langdon & Atkinson (2005) demonstrated that following nutrient enrichment the sensitivity of an assemblage of P. compressa/M. capitata shifted from high to low sensitivity to acidification. Such strong temperature, nutrient and acidification interactions are not well explained simply by invoking changes in carbonate concentrations as the sole causative agent and indicate that the magnitude of the response may be mediated by factors other than [CO$_3^{2-}$].

Low sensitivity to reduced [CO$_3^{2-}$] and/or Ωarag, however, is in disagreement with other experimental studies of the effects of acidification on coral calcification. Some of the discrepancy between the current results for M. auretenra and other experimental studies may be in part due to differences in methodology. Several studies to date have used HCl to mimic the changes induced by CO$_2$ acidification, instead of manipulating pCO$_2$ through the administration of desired amounts of CO$_2$(g). To achieve a given [CO$_3^{2-}$] and Ωarag, HCl acidification yields lower [HCO$_3^-$] and slightly higher pH than CO$_2$ acidification. For example, at 3 x preindustrial pCO$_2$, [HCO$_3^-$] increases ~14% from acidification with CO$_2$(g) (relative to present-day), but only increases ~5% when HCl is used to achieve the same [CO$_3^{2-}$] in a closed system. While these differences are small, they may be enough to influence the magnitude of the response. Close examination of the data from the studies compiled by Langdon & Atkinson (2005) showed that the studies using HCl for acidification had more results in the ‘high sensitivity’ group than studies that used CO$_2$. There is little overlap in species between the data sets, hence species-specific differences cannot be ruled out, but the differences in the magnitude of response suggest that studies utilizing HCl may generally find larger reductions of calcification in corals than those utilizing CO$_2$. In the present study, M. auretenra was able to maintain normal rates of calcification in CO$_2$ acidified seawater. This may be because elevated [HCO$_3^-$] ameliorated any negative affects on calcification associated with reduced
[CO$_3^{2-}$], $\Delta$arag or pH. But it is also possible that $M. auretenra$ is more pH tolerant because it may experience low pH among its crowded branches on a regular basis. The interstices within $M. auretenra$ colonies are habitat for a broad range of microbes and small invertebrates that respire and excrete CO$_2$ into the interstitial seawater. Thus, the proposition is that experimental species and/or methodology employed may affect the magnitude of the observed response.

Importantly, the data showing changes in response to ocean acidification in conjunction with changes in temperature, nutrients or methodology suggest that a predictive model based solely on aragonite saturation is insufficient to predict coral (and other calcifier) calcification responses to ocean acidification caused by CO$_2$ enrichment. Although many studies have reported an empirical relationship between calcification rates and [CO$_3^{2-}$] in the external seawater, a plausible mechanism for the relationship has yet to be determined. Data from the current study and those cited earlier indicate that if such a mechanism does exist it is at the very least influenced by temperature, nutrient supply and HCO$_3^-$ concentrations and is thus not a straightforward predictive tool.

Although there is presently no verified mechanistic basis for the correlation of calcification with aragonite saturation state, the relationship is certainly indicative that changes in ambient seawater chemistry can affect calcification. In agreement with Marubini & Thake (1999), Marubini et al. (2008) and Herfort et al. (2008), the current data support the idea that HCO$_3^-$ plays an important role in determining calcification rates. It is interesting to note that although Marubini et al. (2008) concluded that HCO$_3^-$ enrichment enhanced calcification through increased CO$_3^{2-}$, their low pH, HCO$_3^-$ enriched treatment, supported higher calcification rates than two of the nonenriched treatments despite having lower CO$_3^{2-}$. Herfort et al. (2008) proposed several mechanisms by which increased HCO$_3^-$ resulting from CO$_2$ acidification could stimulate calcification including enhanced transport of HCO$_3^-$ and Ca$^{2+}$ to the calcifying fluid, regulation of cytosol pH or stimulation of photosynthesis, which can stimulate calcification. The linkage of calcification and photosynthesis may also play a role in the response to ocean acidification. Indeed, McConnaughey et al. (2000) suggest that the ratio of calcification to photosynthesis in symbiotic corals is governed by the ratio of alkalinity to acidity in the ambient seawater with an approximately linear relationship over a pH range of 7.6–8.3. Since ocean acidity is changing on a much faster time scale than alkalinity, less calcification will be needed to obtain the same particular benefit in terms of carbon supply for photosynthesis. The study of Schneider & Erez (2006) may have demonstrated this effect. They found no variation in photosynthesis with changes in pH, but a strong positive correlation of calcification with internal pH calculated as an offset of ambient seawater pH. The substantial increase in [H$^+$] associated with ocean acidification may also negatively affect calcification, depending on a coral’s capacity to remove H$^+$ from the site of calcification and expel them into the external environment. If the increase in seawater [H$^+$] impedes H$^+$ removal it may result in reduced pH in the subcalicoblastic space, acidosis in the calicoblastic cells, or reduced calcium transport to the subcalicoblastic space, since Ca$^{2+}$ and H$^+$ transport are coupled in the calicoblastic epithelium (Zoccola et al., 2004). These mechanisms are not mutually exclusive and any of them, or a combination thereof, might affect coral calcification. The overall calcification response to ocean acidification would then be the result of the opposing effects of increased [HCO$_3^-$] and [H$^+$] working against each other.

Changes in ocean chemistry due to CO$_2$ enrichment are already being measured (e.g. Bates, 2001; Brix et al., 2004) and our knowledge of ocean chemistry is such that predictions of future ocean chemistry are robust (e.g. Caldeira & Wickett, 2003). Given the confidence in our ability to predict future ocean chemistry, an empirical relationship between aragonite saturation and coral calcification is very appealing in its simplicity for predicting future calcification rates in response to ocean acidification. However, data from this study and others point to inconsistencies in the aragonite saturation state model, but confirm that changes in seawater chemistry do affect calcification rates. Some of the differences in response may be species dependent, but the inconsistencies among studies also point to a lack of full understanding of the physiological processes that drive coral calcification. If we truly wish to decipher the response of coral calcification to ocean acidification a firmer grasp of the biological component of biomineralization is paramount.

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Supporting Information
Additional Supporting Information may be found in the online version of this article:

Table S1. Summary of the incubations performed in this study to test the effects of altered seawater chemistry on Madracis auretena calcification rates. Data are mean ± SD for the initial and final pH4 (n = 6), the initial TA (n = 3) and individual G (calculated from ᴅTA) for each incubation.

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