Coral Reefs (2017) 36:13–25
DOI 10.1007/s00338-016-1507-z

REPORT

Coral calcification under environmental change: a direct comparison of the alkalinity anomaly and buoyant weight techniques

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Received: 17 December 2015 / Accepted: 24 September 2016 / Published online: 4 October 2016

Abstract Two primary methods—the buoyant weight (BW) and alkalinity anomaly (AA) techniques—are currently used to quantify net calcification rates (G) in scleractinian corals. However, it remains unclear whether they are directly comparable since the few method comparisons conducted to date have produced inconsistent results. Further, such a comparison has not been made for tropical corals. We directly compared G_{BW} and G_{AA} in four tropical and one temperate coral species cultured under various pCO_2, temperature, and nutrient conditions. A range of protocols for conducting alkalinity depletion incubations was assessed. For the tropical corals, open-top incubations with manual stirring produced G_{AA} that were highly correlated with and not significantly different from G_{BW}. Similarly, G_{AA} of the temperate coral was not significantly different from G_{BW} when incubations provided water motion using a pump, but were significantly lower than G_{BW} by 16% when water motion was primarily created by aeration. This shows that the two techniques can produce comparable calcification rates in corals but only when alkalinity depletion incubations are conducted under specific conditions. General recommendations for incubation protocols are made, especially regarding adequate water motion and incubation times. Further, the re-analysis of published data highlights the importance of using appropriate regression statistics when both variables are random and measured with error. Overall, we recommend the AA technique for investigations of community and short-term day versus night calcification, and the BW technique to measure organism calcification rates integrated over longer timescales due to practical limitations of both methods. Our findings will facilitate the direct comparison of studies measuring coral calcification using either method and thus have important implications for the fields

Communicated by Biology Editor Prof. Mark R. Patterson

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Electronic supplementary material The online version of this article (doi:10.1007/s00338-016-1507-z) contains supplementary material, which is available to authorized users.

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of ocean acidification research and coral biology in general.

Keywords Calcification · Buoyant weight · Alkalinity anomaly · Method comparison · Ocean acidification · Incubation

Introduction

There is a pressing need to study coral calcification in the context of current global climate change and ocean acidification (Kleypas et al. 1999; Hoegh-Guldberg et al. 2007; Cantin et al. 2010; Chan and Connolly 2013). Dissolution of atmospheric CO_2 into the surface ocean has caused both decreases in seawater pH (i.e., an increase in proton concentration) and aragonite saturation state (Ω_{arag}) (Caldeira and Wickett 2003; Sabine et al. 2004), which is commonly referred to as ocean acidification (OA). OA leads to lower calcification rates in many, though not all, species of corals (e.g., Langdon et al. 2000; Marubini et al. 2001, 2003; Schoepf et al. 2013; Comeau et al. 2014a). However, it has become increasingly clear that the response of coral calcification to OA varies significantly among studies (Pandolfi et al. 2011; Chan and Connolly 2013), and that the method used to measure calcification rates can introduce a significant bias (Chan and Connolly 2013). Specifically, Chan and Connolly (2013) found that studies employing buoyant weighing observed significantly smaller decreases in coral calcification per unit Ω_{arag} ($\sim$10%) compared with studies using the alkalinity anomaly technique ($\sim$25%). This suggests that studies using different methods to measure coral calcification rates may not be comparable, thus making it difficult to estimate the true sensitivity of coral calcification to OA and to accurately incorporate coral growth rates into reef accretion models under future OA scenarios.

Two primary techniques are used to evaluate the rate of net calcification (i.e., gross calcification minus dissolution, G) in corals: the buoyant weight and the alkalinity anomaly techniques. The buoyant weight (BW) method was first developed in the 1960s and is based on Archimedes' principle (Bak 1973; Jokiel et al. 1978). It involves weighing a live coral fragment before and after a certain time period while suspended in seawater of known density (calculated from salinity and temperature). Important assumptions of this method are that (1) the coral skeleton consists entirely of aragonite, (2) living coral tissue and mucus have the same density as seawater, (3) any cryptic coral-associated fauna consists largely of neutrally buoyant tissue and thus does not affect the buoyant weight, and (4) voids and spaces within the porous skeleton are filled with liquid of the same density as the buoyant medium (Jokiel et al. 1978). Spencer Davies (1989) demonstrated that in some coral species, tissue weight can contribute up to 5% to the BW and accuracy can therefore be improved by applying a correction.

The BW technique is widely used as it is a simple, inexpensive, and non-destructive method that directly measures the weight of aragonite. It is easily used in the field and the laboratory, and allows for repeated measurements of the same fragment over time. Although this method may measure coral calcification over as little as 12 h (Jokiel et al. 1978; Spencer Davies 1989), it is typically employed on much longer timescales (weeks to months) and then provides an integrated measure of day and night calcification over extended time periods.

The second widely used method to measure net calcification in corals and coral reef communities is the alkalinity anomaly (AA) technique (Smith and Key 1975; Smith and Kinsey 1978). In a laboratory setting, this method involves incubating a live coral in a small volume of seawater for a short period of time (typically hours) and measuring the resulting change in total alkalinity (A_T). For each mole of $CaCO_3$ precipitated, A_T is lowered by two molar equivalents based on the following reaction:

$$Ca^{2+} + 2HCO_3^- = CaCO_3 + CO_2 + H_2O \quad (1)$$

The amount of alkalinity loss can then be converted to the weight of $CaCO_3$ precipitated. For detailed practical guidelines, see Riebesell et al. (2010). Significant advantages of the AA technique include the ability to easily compare day and night calcification rates as well as to measure community calcification over timescales of days to weeks or even months when water residence time is taken into account (Broecker and Takahashi 1966; Smith and Pesret 1974; Langdon et al. 2000; Anthony et al. 2011; Comeau et al. 2013, 2014b).

The AA technique assumes that (1) changes in A_T due to nutrient assimilation and release or sulfate reduction are negligible, (2) only dissolution and calcification affect A_T, and (3) the removal of ionic CO_2 species during photosynthesis, and vice versa during respiration, is rapidly balanced by production of other anions with equivalent charge so that A_T is not affected (Chisholm and Gattuso 1991). Chisholm and Gattuso (1991) demonstrated experimentally that these assumptions are fundamentally valid and that corrections to account for changes in nutrient concentration over the course of the incubations are not required in symbiotic corals (see also Gazeau et al. 2015). Similarly, coral calcification rates obtained using this technique are highly correlated with rates determined using radioisotopes (^{45}Ca) (Tambutté et al. 1995) and the calcium anomaly technique Gazeau et al. (2015), and AA- and calcium anomaly-based rates are not significantly different (Gazeau et al. 2015). With respect to coral reef

communities, a recent study showed that the AA technique agrees with calcium uptake (in a ratio of 2:1) in a coral-only system but underestimates community calcification due to benthic alkalinity release (Murillo et al. 2014).

Although the BW and AA methods should theoretically result in comparable calcification rates, a meta-analysis concluded that significantly different responses to OA were observed depending on which method was used to measure coral calcification (Chan and Connolly 2013). It was hypothesized that this could be due to the fact that the BW method typically integrates over both light and dark calcification on long timescales, whereas alkalinity depletion incubations are typically performed over much shorter intervals (hours to a few days). To date, very few studies have attempted a direct comparison of the two methods (Table 1), and the existing results are inconsistent (Steller et al. 2007; Holcomb et al. 2010; Rodolfo-Metalpa et al. 2010; Maier et al. 2013). Holcomb et al. (2010) found good agreement between the two methods in a temperate coral under long-term CO_2 and nutrient enrichment. Similarly, Maier et al. (2013) showed that for two cold-water coral species, the two methods provided comparable calcification rates in a 9-month OA experiment. These two studies were unusual in that corals were incubated for two full days instead of only a few hours. In contrast, the AA technique significantly underestimated calcification rates in a coralline alga (Steller et al. 2007) and a temperate coral (Rodolfo-Metalpa et al. 2010).

Given these inconsistent findings and the fact that none of these studies were conducted with tropical coral species, symbiotic versus non-symbiotic corals, or in combination with elevated temperature, further studies directly comparing the two methods are required. This will further improve our understanding of how sensitive coral calcification is to a variety of environmental factors including OA. Here, we used data from two different experiments to directly compare the BW and AA techniques in four tropical and one temperate coral species cultured under various combinations of pCO_2, temperature, and nutrients. Consequently, different alkalinity incubation setups were compared (Fig. 1). We examined whether coral calcification rates were independent of the measurement method, and whether this was consistent across a large range of coral species and environmental conditions.

Materials and methods

The two techniques to measure coral calcification rates were compared using data from two separate experiments (Holcomb et al. 2012; Schoepf et al. 2013), which are described below. The two experiments were conducted at different times, and it was therefore not planned a priori to combine their data for this method comparison. However, these data provided an opportunity to analyze the most comprehensive data set of paired BW- and AA-based calcification rates to date and allowed for a more comprehensive comparison of various incubation techniques.

Experiment 1: calcification rates of four tropical coral species at various pCO_2 and temperature levels

This experiment was conducted at Reef Systems Coral Farm (New Albany, OH, USA) in July/August 2011 and is described in detail in Schoepf et al. (2013). In brief, six colonies of the Pacific coral species *Acropora millepora*, *Montipora monasteriata*, *Pocillopora damicornis*, and *Turbinaria reniformis* were fragmented into small pieces ($\sim$70–450 cm^2) and randomly assigned to six treatments, which consisted of two temperature regimes (26.5 and 29.0 °C averaged over the entire experiment) crossed with three pCO_2 levels (382, 607, and 741 µatm). Seawater pCO_2 was controlled by bubbling in pure CO_2, CO_2-free air, or ambient air. Temperature and pCO_2 were raised gradually over several days to prevent heat or pCO_2 shock. Corals were grown under experimental treatments for 24 d on a 9:15-h light:dark cycle (275 µmol quanta m^{-2} s^{-1}) and fed every 3 d with brine shrimp. Water motion within each 57-L aquarium was provided by powerheads (Accela SPI-1000, $\sim$1000 L h^{-1}; Fig. 1a). Each fragment was attached to a pre-labeled PVC tile, which was thoroughly cleaned of any fouling material and algae prior to conducting the measurements described below.

Buoyant weight measurements

Coral fragments were weighed using the BW technique (Jokiel et al. 1978) at the beginning and end of the experiment to calculate daily calcification rates (G_{BW}) in mg d^{-1} (see Electronic Supplementary Material, ESM, for more details).

Open-top alkalinity depletion incubations (day and night): AA method 1

Open-top incubations were performed during the day and at night on the same set of corals as above at various, random time points during the experiment (2 incubations × 6 fragments × 6 treatments × 4 species = 288 incubations). Each fragment was incubated in individual open-top, wide-mouthed plastic chambers (diameter 10.5 cm, depth 17 cm) filled with treatment-specific seawater (1.5 L volume) for 1.5 h (Fig. 1a). The chambers were partially immersed in their respective experimental tanks to maintain constant temperature and to receive the same light levels as during the general culturing setup. A

Table 1 Summary of studies that have compared the buoyant weight and alkalinity anomaly techniques to measure coral calcification rate

Species	Organism size	Seawater volume (L)	Incubation time (h)	Open or closed	Stirring method	pCO_2 control	Nutrient corrected	Methods agree?
Coralline algae								
Lithophyllum margaritae[a]	10 g	1	60	Open	Stir bars	No	No	No
Cold-water corals								
Lophelia pertusa, Madrepora oculata[b]	15–90 polyps	0.2 or 0.7	48	Open	Aeration	Yes	Yes	Yes[b]
								No[e]
Temperate corals								
Cladocora caespitosa[c]	10–20 polyps	0.05	5 (light and dark)	Closed	Stir bars	No	No	No
Astrangia poculata[d]	2.6 ± 1.3 g (13 ± 3 cm^2)	0.8	48	Open (lid present)	Aeration	Yes	No	Yes[d]
								No[e]
Astrangia poculata[e] (AA method 2)	2 ± 1 g	0.5	8–11 (day and night)	Open (lid present)	Aeration	Yes	No	No[f]
Astrangia poculata[e] (AA method 3)	2 ± 1 g	0.93	5–6 (day and night)	Closed	Pump	No	No	Yes[f]
Tropical corals								
Acropora millepora, Pocillopora damicornis, Montipora monasteriata, Turbinaria reniformis[e] (AA method 1)	~70–450 cm^2	1.5	1.5 (day and night)	Open	Manual stirring every 5–10 min	No	No	Yes

Methodological details are given for the alkalinity depletion incubations. Errors represent standard deviations

[a] Steller et al. (2007)

[b] Maier et al. (2013)

[c] Rodolfo-Metalpa et al. (2010)

[d] Holcomb et al. (2010)

[e] This study

[f] When symbiotic and non-symbiotic corals were pooled

water sample was taken just before the onset of the incubation from each treatment tank for pH and A_T analysis. Seawater in the incubation chambers was stirred manually every 5–10 min to minimize the effect of stagnant water on calcification rates. This method of providing water circulation was chosen due to logistic constraints in order to accommodate AA incubations on all fragments of all four species in this experiment ($n = 288$ incubations). At the end of each incubation, a water sample was taken from the chamber using a screw-top low-density polyethylene bottle for A_T titration. Incubations in the light were done randomly during the day, while the dark incubations were initiated at least half an hour after the artificial lights were turned off in the evening. Four random control incubations (i.e., without a coral fragment) were conducted throughout the experiment and showed that alkalinity gain due to evaporation was 7 ± 1 (SD) μM over the 1.5-h incubation. To correct for A_T changes due to evaporation, this value was thus subtracted from all measured A_T changes (ΔA_T) for all incubations with the presence of coral fragments. Further details regarding the analysis of pH and A_T and estimated changes in Ω_{arag} over the course of the incubations are given in the ESM.

Daily calcification rates (mg d^{-1}) obtained using open-top incubations (G_{AA1}) were then calculated using the following approach:

$$G_{AA1} = (\Delta A_{T-D} \times t_D + \Delta A_{T-L} \times t_L) \times \rho \times \frac{V}{(2 \times 10)} \quad (2)$$

where ΔA_T is the hourly alkalinity consumption rate measured over the course of the incubation (μmol kg^{-1} h^{-1}), subscripts D and L represent dark and light, respectively, t is hours in a day (9 h light, 15 h darkness), ρ is the density of seawater, and V is the volume of the incubation water minus the volume of the coral fragment in liters. Rates were not nutrient-corrected because changes in A_T

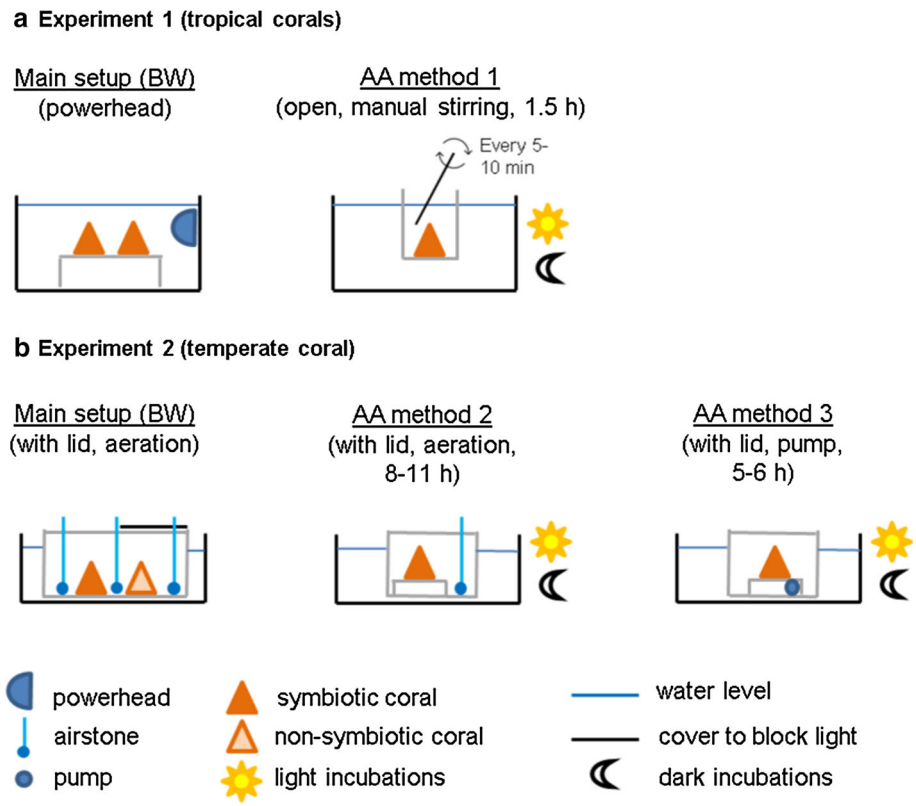

Fig. 1 Schematic representation of the main culturing setup (as represented by buoyant weight-based calcification rates) and various alkalinity depletion incubations for (**a**) four tropical corals and (**b**) a temperate coral. For alkalinity anomaly methods 2 and 3, the setup was the same for both symbiotic and non-symbiotic corals. *BW* buoyant weight, *AA* alkalinity anomaly. Drawings are not to scale

due to nutrient release and consumption are negligible compared to changes in A_T due to calcification in tropical corals (Chisholm and Gattuso 1991).

Experiment 2: calcification rates of a temperate coral at various pCO_2, nutrient, and temperature levels

This experiment was conducted at the Woods Hole Oceanographic Institution (WHOI, Woods Hole, MA, USA) in 2008 and is described in detail in Holcomb et al. (2012). Briefly, four non-symbiotic (white) and four symbiotic (brown) colonies of the temperate coral *Astrangia poculata* were collected from shallow depth from the WHOI pier, divided into fragments (2 ± 1 g), and mounted onto acrylic slides. Surface area was not determined for these corals but estimated to be in the range of 10–16 cm^2 based on measured surface areas from a previous similar experiment (Holcomb et al. 2010). Corals were maintained in flow-through tanks with filtered seawater with half of each tank being exposed to light (for symbiotic corals) and the other half kept dark (for non-symbiotic corals) (Fig. 1b).

The experiment was carried out in four steps: (1) an initial acclimation phase during which corals were gradually transitioned to experimental temperatures; (2) a pre-treatment phase in which baseline growth rates were established for each coral at the treatment temperature; (3) a second acclimation phase during which corals were gradually transitioned to experimental nutrient and pCO_2 levels; and (4) a treatment phase during which corals were maintained under the desired treatment conditions for 6 months. During the treatment phase, two temperature levels (16 and 24 °C) were crossed with two pCO_2 levels (373 and 801 µatm) and two nutrient levels (ambient 0.6 ± 0.5 µmol L^{-1} NH_4^+, 3.3 ± 0.8 µmol L^{-1} NO_3^-, and 0.6 ± 0.1 µmol L^{-1} PO_4^{3-}, elevated 0.3 ± 0.2 µmol L^{-1} NH_4^+, 8.7 ± 1.2 µmol L^{-1} NO_3^-, and 0.9 ± 0.1 µmol L^{-1} PO_4^{3-}). Seawater pCO_2 was controlled by bubbling in the respective air/CO_2 mixture. For the symbiotic corals, light levels of 15–24 µmol quanta m^{-2} s^{-1} were provided on a 12:12-h light/dark cycle and are representative of the in situ light levels at the collection site. Some stray light reached the non-symbiotic corals during the day but was <10 µmol quanta m^{-2} s^{-1}. Corals were fed daily with freshly hatched brine shrimp. Water motion in the 1.9-L tanks was provided by three airstones. All corals and their slides were carefully cleaned of algae before analysis.

Buoyant weight measurements

Buoyant weight measurements were made ~1 and ~6 weeks after the treatment phase was established to calculate daily calcification rates (G_{BW}, mg d^{-1}; see ESM for details).

Alkalinity depletion incubations with gas exchange (day and night): AA method 2

Covered incubations with aeration were conducted ~1.5 weeks after the treatment phase was established during the day and at night by incubating the same corals in 1-L containers for 8–11 h each (2 incubations × 8 or 10 fragments × 8 treatments × 1 species = 144 incubations) (Fig. 1b). Plastic lids covered the containers to reduce air exchange with the overlying atmosphere, thus increasing the contact time between the bubbled gas and the water. However, the lids were not air-tight, and bubbled gasses could thus escape. Approx. 0.5 L of the treatment water (precise amount weighed) was used for both coral and control incubations (blank slide and water only). Water temperature was kept constant via a water bath, and treatment pCO_2 was maintained by bubbling the appropriate treatment gas with airstones, which also provided the main source of water motion. For symbiotic corals, incubations were conducted in the light during the day at light levels that were similar to those in the general culturing setup, and in the darkness at night. For non-symbiotic corals, incubations were conducted without lights during the night and under very low light levels (<10 µmol quanta m^{-2} s^{-1}) during the day; therefore, light levels during the incubations were similar to those in the general culturing setup. Corals were allowed to acclimate to the incubation containers for ~15 min. Dark incubations lasted from ~midnight until 0930 h, then the lights were turned on, and light incubations were started immediately and continued until ~1800 h. A_T samples were taken from the incubation water at the beginning and the end of each day and night incubation, respectively. Seawater pH_{NBS} and salinity were also measured at the end of each incubation.

Alkalinity depletion rates were corrected for alkalinity gain due to evaporation based on the change in container mass (assuming linear rates) using a balance (0.01 g precision) as this provided greater precision than the probe used to measure salinity (cumulative measurement error of ~0.1 g). Typically, a loss of ~2 g was observed due to evaporation. In addition, alkalinity depletion rates were corrected for background changes in A_T due to processes other than evaporation, which were measured during control incubations without coral. However, changes in A_T due to evaporation were greater than background changes because dry air was used for bubbling. Further, background alkalinity consumption rates were invariably low relative to coral calcification rates. A nutrient correction was not applied (Holcomb et al. 2010; Gazeau et al. 2015). Daily calcification rates (mg d^{-1}) obtained using incubations with gas exchange (G_{AA2}) were calculated as follows:

$$\Delta A_{TL-\text{control}} = A_{T1} \times M_{SW1} - A_{T2} \times M_{SW2} \quad (3)$$

$$\Delta A_{TD-\text{control}} = A_{T3} \times M_{SW3} - A_{T4} \times M_{SW4} \quad (4)$$

$$G_{AA2} = \left\{ \left[\frac{(A_{T1} \times M_{SW1} - A_{T2} \times M_{SW2}) - \Delta A_{TL-\text{control}}}{t_L} \right] \times 12\text{ h} + \left[\frac{(A_{T3} \times M_{SW3} - A_{T4} \times M_{SW4}) - \Delta A_{TD-\text{control}}}{t_D} \right] \times 12\text{ h} \right\} \times \frac{1}{(2 \times 10)} \quad (5)$$

where $\Delta A_{TL-\text{control}}$ and $\Delta A_{TD-\text{control}}$ are the change in A_T during light and dark control incubations, respectively; A_{T1}/A_{T3} and A_{T2}/A_{T4} are A_T at the beginning and end of the light or dark incubation (µmol kg^{-1}), respectively; M_{SW1}/M_{SW3} and M_{SW2}/M_{SW4} are the mass of seawater at the beginning and end of the light or dark incubation (kg), respectively; and t_L and t_D are the incubation duration (h) in the light and dark, respectively.

Alkalinity depletion incubations without gas exchange (day and night): AA method 3

Covered incubations were conducted 3–5.5 weeks after the treatment phase was established during day and night on the same corals using submersible pumps to avoid gas exchange (2 incubations × 8 or 10 fragments × 8 treatments × 1 species = 144 incubations) (Fig. 1b). Small aquarium pumps (AZOO, Taiwan) were sealed inside 1-L containers with plastic lids providing flow rates of ~90 L h^{-1}. A tray was used to hold corals ~2 cm off the bottom of the container. Great care was taken to avoid any air bubbles being trapped within the container. Total water volume was ~0.93 L, and placement of containers within a water bath ensured constant temperature. For symbiotic corals, incubations were conducted in the light during the day at light levels that were similar to those in the general culturing setup and in the darkness at night. For non-symbiotic corals, incubations were conducted without lights during the night and under very low light levels (<10 µmol quanta m^{-2} s^{-1}) during the day; therefore, light levels during the incubations were similar to those in the general culturing setup. Chambers with a coral, blank slide, or water only were incubated for 5–6 h in the light during the day and in the dark at night. At the end of each incubation, seawater pH_{NBS}, conductivity, and temperature were measured, and samples for A_T were collected. After a ~5-h light incubation, containers were then left open for ~1 h, bubbled with the respective treatment pCO_2, and 90 mL of treatment water added before starting the dark incubations to re-establish treatment conditions. A nutrient correction was not applied (Holcomb et al. 2010; Gazeau et al. 2015). Daily calcification rates (mg d^{-1}) obtained

using incubations without gas exchange are referred to as G_{AA3} and were calculated using Eqs. 3–5.

Statistical analyses

Model II linear regression (Legendre and Legendre 1998) was used to assess the relationship between calcification rates obtained using the different methods because all calcification rates represented random variables and were measured with error for both techniques. In such cases, model I regression using least squares underestimates the slope of the linear relationship (Legendre and Legendre 1998). Although model I regression can nevertheless be used under these circumstances when prediction is the main goal of the study, model II regression should be used when describing the true nature of the relationship between the two variables is the main focus (Legendre and Legendre 1998; Quinn and Keough 2002). Ranged major axis (RMA) regression was used since variables were originally measured in different units (Legendre and Legendre 1998). Calcification rates obtained using each of the three different AA methods were compared to the calcification rates obtained using the BW technique. This was done using multiple subsets of the data by pooling data from both experiments, for tropical and temperate corals, and for each individual coral species and treatment level, respectively, to evaluate the robustness of a given relationship obtained for all corals (or all tropical and all temperature corals). Further, published G_{BW} and G_{AA} for the temperate coral *Astrangia poculata* (Holcomb et al. 2010) and the cold-water corals *Madrepora oculata* and *Lophelia pertusa* (Maier et al. 2013) were re-analyzed using RMA regression to facilitate comparison with this study. Regressions were computed using the "lmodel2" package with 99 permutations in R software (version 3.1.2). p values ≤ 0.05 were considered significant.

Results

Changes in carbonate chemistry during AA incubations

In experiment 1, A_T during the open-top incubations (AA method 1) changed on average by 0.9 ± 0.9% standard deviation (22 ± 20 µmol kg^{-1}), ranging from 0.3% (7 µmol kg^{-1}) increase in the dark (presumably due to slight dissolution) to 3.7% (88 µmol kg^{-1}) reduction in the light (Table 2). The estimated average decrease in Ω_{arag} was 0.06 units (Table 2).

In experiment 2, pH decreased on average by 0.04 units over the course of the incubations with gas exchange (AA method 2), whereas it typically decreased by 0.09 units during incubation without gas exchange (AA method 3) (Table 2). Total alkalinity decreased on average by <4% during AA method 2 and 3 incubations (79 and 25 µmol kg^{-1}, respectively) (Table 2). The resulting average decrease in Ω_{arag} was 0.20 and 0.33 units for the two AA methods, respectively (Table 2).

Comparison of BW technique and AA methods 1 and 2 (open-top/with gas exchange)

In all four tropical coral species, G_{BW} and G_{AA1} were strongly correlated and not significantly different from each other, although all slopes were <1 and the intercept was significantly lower than 0 when all tropical corals were pooled (Table 3a; Fig. 2). The correlation was generally strongest for *P. damicornis* ($R^2 = 0.79$; Fig. 2) and weakest for *Montipora monasteriata* ($R^2 = 0.43$; Fig. 2). In contrast, when the data for all five coral species examined in this study were pooled across both experiments, G_{AA1+2} were strongly correlated with G_{BW} ($R^2 = 0.77$; Table 3a) but nevertheless significantly lower (−21%) than G_{BW} (Table 3a). This trend was particularly evident for the temperate coral *A. poculata* when symbiotic and non-symbiotic corals were pooled (Table 3a; Fig. 3a). G_{AA2} were also significantly lower than G_{BW} in symbiotic *A. poculata* only, but did not differ significantly in non-symbiotic corals (Table 3a).

Treatment-specific comparisons pooled for all tropical species (Experiment 1) also showed that G_{AA1} were not significantly different from G_{BW} with the exception of 382 µatm, where G_{AA1} were significantly lower than G_{BW} (−23%) (Table 3b). Treatment-specific comparisons for temperate *A. poculata* (Experiment 2) were largely consistent with the trends observed when all treatments were pooled. All pCO_2, temperature, and nutrient treatments had slopes <1, and the majority of them were significantly different from 1 (Table 3c).

Comparison of BW technique and AA method 3 (without gas exchange)

G_{BW} was strongly correlated ($R^2 = 0.80$) with G_{AA3} in all *A. poculata* corals (Experiment 2), and the two estimates were not significantly different (Table 4a; Fig. 3b). This was further confirmed by treatment-specific comparisons, although G_{AA3} was significantly higher (+15%) than G_{BW} at 373 µatm (Table 4b). Further, non-symbiotic *A. poculata* corals also had significantly higher G_{AA3} than G_{BW} (+13%; Table 4a).

Table 2 Changes in pH, total alkalinity (A_T), oxygen concentration and aragonite saturation state (Ω_{arag}) over the course of alkalinity depletion incubations for various alkalinity anomaly (AA) methods relative to tank conditions

	pH (NBS scale)	A_T (μmol kg^{-1})	Ω^a_{arag}
Experiment 1—open-top incubations (AA method 1)			
Max. change	n/a	−88	−0.49
Average ($n = 284$)	n/a	−22	−0.06
SD	n/a	20	0.14
Experiment 2—incubations with gas exchange (AA method 2)			
Max. change	−0.13	−191	−0.49
Average ($n = 72$)	−0.04	−79	−0.20
SD	0.04	48	0.13
Experiment 2-incubations without gas exchange (AA method 3)			
Max. change	−0.19	−63	−0.81
Average ($n = 72$)	−0.09	−25	−0.33
SD	0.04	17	0.19

[a] Ω_{arag} changes in the open-top incubations (Experiment 1) were estimated using experimentally measured CO_2 gas transfer velocity (see ESM)

Table 3 Results from model II ranged major axis regression analyses comparing calcification rates obtained using the buoyant weight technique and alkalinity anomaly (AA) methods 1 and 2

	AA method	N	R^2	Intercept	Slope	CI 2.5% slope	CI 97.5% slope
(a) All treatments combined (exp. 1 and 2)							
All corals (experiment 1 + 2)	1 + 2	210	0.77	−1.15	0.79	**0.74**	**0.85**
Tropical corals (experiment 1)	1	138	0.61	−4.51*	0.88	0.77	1.01
Acropora millepora	1	33	0.57	−1.35	0.81	0.58	1.12
Pocillopora damicornis	1	35	0.79	−2.72	0.91	0.76	1.09
Montipora monasteriata	1	36	0.43	−7.47	0.86	0.55	1.27
Turbinaria reniformis	1	34	0.56	−7.95	0.99	0.73	1.39
Temperate corals (experiment 2): all *Astrangia poculata*	2	72	0.84	−0.04	0.84	**0.75**	**0.93**
Symbiotic *A. poculata* only	2	36	0.80	−0.02	0.79	**0.66**	**0.94**
Non-symbiotic *A. poculata* only	2	36	0.90	−0.07	0.90	0.80	1.01
(b) Treatment-specific comparisons: tropical corals (exp. 1)							
382 μatm	1	48	0.69	0.59	0.77	**0.63**	**0.94**
607 μatm	1	45	0.51	−6.44	0.86	0.63	1.17
741 μatm	1	45	0.53	−6.61	1.00	0.75	1.36
26.5 °C	1	70	0.66	−2.55	0.86	0.72	1.03
29.0 °C	1	68	0.60	−5.24	0.85	0.69	1.04
(c) Treatment-specific comparisons: temperate coral (exp. 2)							
373 μatm	2	36	0.88	−0.12	0.88	**0.77**	**0.99**
801 μatm	2	36	0.76	0.13	0.76	**0.62**	**0.93**
16 °C	2	32	0.84	0.04	0.75	**0.64**	**0.88**
24 °C	2	40	0.82	0.25	0.81	**0.70**	**0.95**
Ambient nutrients	2	36	0.82	−0.31	0.92	0.78	1.09
Elevated nutrients	2	36	0.86	0.17	0.78	**0.68**	**0.90**

All regressions and one-tailed permutational p values for the slope were statistically significant at $p < 0.05$. 95% confidence intervals (CI) are given for the regression slope. CIs of the slope that were significantly different from 0 or 1 are highlighted in bold

* Intercepts that were significantly different from 0

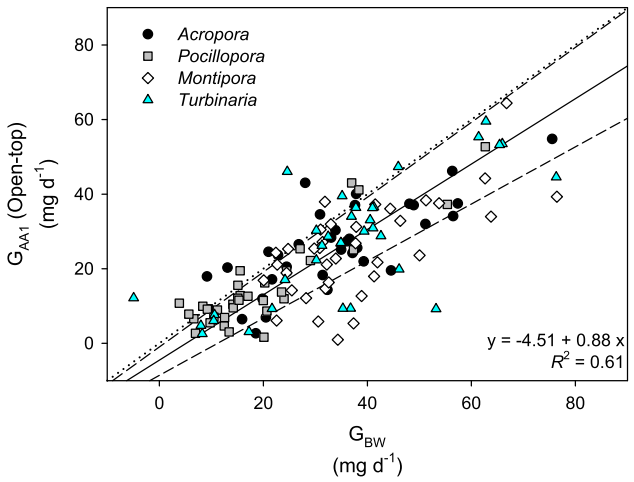

Fig. 2 Comparison of calcification rates in four tropical coral species measured with the buoyant weight (G_{BW}) and alkalinity anomaly method using open-top incubations (G_{AA1}). Incubations were conducted during day and night. *Symbols* represent individual coral fragments. The *solid line* indicates the ranged major axis regression line, and *dashed lines* represent 2.5 and 97.5% confidence intervals (CIs), respectively. The *dotted line* indicates perfect agreement between the two methods

Re-analysis of published method comparisons

A re-analysis of the calcification data from Holcomb et al. (2010) showed that incubations of the temperate coral *A. poculata* using a design similar to AA method 2 (see Table 1 for details) resulted in significantly lower G_{AA} than G_{BW}: $G_{AA} = 0.98 + 0.67\ G_{BW}$ ($R^2 = 0.52$, $p < 0.001$, 95% CIs for slope 0.45–0.99). Similarly, re-analysis of the data from Maier et al. (2013) showed that G_{AA} of the Mediterranean cold-water corals *Madrepora oculata* and *L. pertusa* were significantly lower than G_{BW} in both species: *M. oculata*: $G_{AA} = 0.01 + 0.53\ G_{BW}$, $R^2 = 0.78$, $p < 0.001$, 95% CIs for slope 0.41–0.69; *L. pertusa*: $G_{AA} = 0.01 + 0.50\ G_{BW}$, $R^2 = 0.75$, $p < 0.001$, 95% CIs for slope 0.36–0.71 (see Table 1 for details regarding the incubation design).

Discussion

Agreement between the buoyant weight and alkalinity anomaly techniques

Two of the AA incubation protocols investigated here produced calcification rates that were strongly correlated and not significantly different from calcification rates determined by the BW method on the same set of corals: (1) open-top incubations of four tropical coral species that used manual stirring to create water motion (AA method 1); and (2) incubations of the temperate coral *A. poculata* that were conducted without gas exchange but with submersed pumps (AA method 3). The same findings were typically also observed when treatments instead of species were pooled for these method comparisons. This demonstrates that comparable calcification rates can be measured using both techniques in scleractinian corals, even when AA incubations are separated by several weeks.

Nevertheless, the strength of the relationship between AA- and BW-based calcification rates differed somewhat between species and AA methods. For example, agreement between the two techniques was less robust in the tropical coral species where R^2 values were lower and slopes generally tended to be <1 (though they were not significantly different from 1) compared to temperate *A. poculata*. Furthermore, the intercept was significantly lower than 0 when all tropical corals were pooled, indicating a

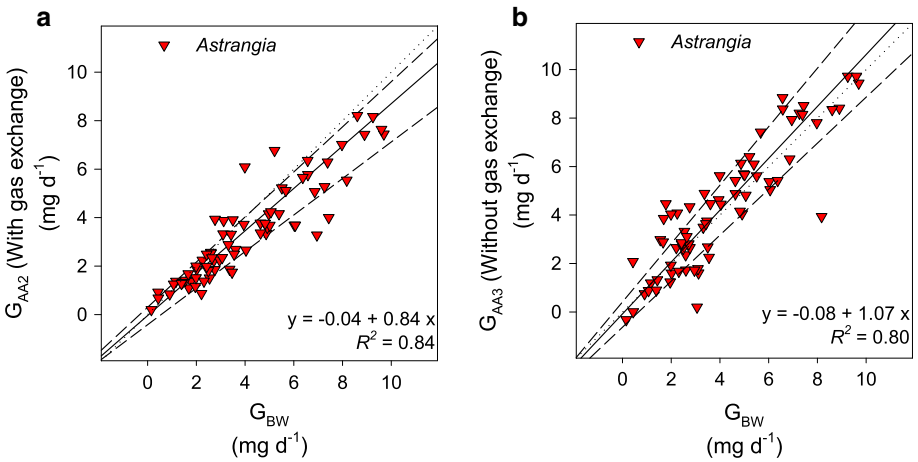

Fig. 3 Comparison of calcification rates measured with the buoyant weight (G_{BW}) and alkalinity anomaly method using incubations with (**a**) and without (**b**) gas exchange (G_{AA2} and G_{AA3}, respectively) in the temperate coral *Astrangia poculata*. Incubations were conducted during day and night. *Symbols* represent individual coral fragments. The *solid line* indicates the ranged major axis regression line, and *dashed lines* represent 2.5 and 97.5% confidence intervals (CIs), respectively. The *dotted line* indicates perfect agreement between the two methods

Table 4 Results from model II ranged major axis regression analyses comparing calcification rates obtained using the buoyant weight technique and alkalinity anomaly method 3

	AA method	*N*	R^2	Intercept	Slope	CI 2.5% slope	CI 97.5% slope
(a) All treatments combined (experiment 2)							
Temperate corals (experiment 2): all *Astrangia poculata*	3	72	0.80	−0.08	1.07	0.95	1.20
Symbiotic *A. poculata* only	3	36	0.75	0.26	1.00	0.81	1.22
Non-symbiotic *A. poculata* only	3	36	0.85	−0.32	1.13	**1.13**	**1.31**
(b) Treatment-specific comparisons: temperate coral (experiment 2)							
373 µatm	3	36	0.88	−0.50	1.15	**1.01**	**1.31**
801 µatm	3	36	0.68	0.29	0.98	0.77	1.25
16 °C	3	32	0.59	−0.02	1.02	0.74	1.40
24 °C	3	40	0.82	0.06	1.06	0.90	1.24
Ambient nutrients	3	36	0.82	0.39	1.08	0.91	1.27
Elevated nutrients	3	36	0.84	−0.58	1.07	0.92	1.25

All regressions and one-tailed permutational *p* values for the slope were statistically significant at $p < 0.05$. 95% confidence intervals (CI) are given for the regression slope. CIs of the slope that were significantly different from 0 or 1 are highlighted in bold. None of the intercepts were significantly different from 0

systematic offset between the two methods. In part, this could have been due to smaller sample sizes for each tropical species compared to *A. poculata*. However, this also suggests that some aspects of AA method 1 were not as ideal as AA method 3 and/or that some coral species (e.g., *Montipora monasteriata*) have more variable calcification rates over time than others (e.g., *P. damicornis*). In such cases, it seems that the AA technique tends to underestimate, rather than overestimate, true calcification rates.

Disagreement between the buoyant weight and alkalinity anomaly techniques

AA method 2 (covered incubations with airstones for gas exchange and water motion) resulted in significant differences between the BW and AA techniques in the temperate coral *A. poculata*. Specifically, G_{AA2} were 16% lower than G_{BW} (Fig. 3a; Table 3a). This appears to contradict a previous comparison for the same coral species (Holcomb et al. 2010), where the two techniques were found to result in comparable calcification rates. In contrast to the present study, Holcomb et al. (2010) used much longer incubation times (48 vs. 8–11 h), but both incubation designs used aeration for pCO_2 control and to create water motion (Table 1). More importantly however, Holcomb et al. (2010) analyzed the data using model I regression with G_{AA} as the independent variable. A re-analysis of their data using RMA regression showed that G_{AA} was in fact also significantly lower (−33%) than G_{BW} and is thus in agreement with this study.

Interestingly, Maier et al. (2013) also performed a method comparison using AA incubations with aeration for the Mediterranean cold-water corals *Madrepora oculata* and *L. pertusa* at two pCO_2 levels. Paired t-tests showed that G_{BW} and G_{AA} were not significantly different in either species. However, it is likely that the statistical power to detect significant differences was low due to high variability in the measured calcification rates. In fact, a re-analysis of their data using RMA regression showed that both species had significantly lower (approximately −50%) G_{AA} than G_{BW}. These findings highlight the importance of using appropriate regression methods when both variables are random and measured with error (Legendre and Legendre 1998).

The fact that some, but not all, AA methods investigated here resulted in good agreement between the BW and AA techniques suggests that methodological differences between incubation methods played a critical role in determining the outcome of the method comparison (see Table 5 for advantages and disadvantages of each AA method). In the temperate coral *A. poculata*, for example, G_{AA2} significantly underestimated G_{BW}, but G_{AA3} did not. Although AA method 3 used shorter incubations (5–6 vs. 8–11 h) and larger incubation volumes (0.9 vs. 0.5 L; see Table 1) than AA method 2, another distinguishing feature between the two methods was water motion. Rather than relying on airstones for water motion, AA method 3 used small pumps with a flow rate of $\sim$90 L h^{-1} (Fig. 1b). Water flow can significantly affect calcification rates by limiting the flux of dissolved substances across the diffusion boundary layer (DBL) (e.g., Jokiel 1978; Dennison and Barnes 1988; Sebens et al. 2003). Furthermore, the AA technique relies on measuring fluxes between the organism and the ambient seawater, which means that if these fluxes

Table 5 Advantages and disadvantages of alkalinity anomaly incubation methods used in this study

Method	Advantages	Disadvantages
AA method 1 (open-top with manual stirring)	Simple, affordable, and fast setup	Non-continuous water motion
	Suited for large number of simultaneous incubations	Carbonate chemistry not controlled*
	Agreement with corresponding G_{BW}	
	Smallest changes in Ω^{a}_{arag}	
AA method 2 (covered container with aeration)	Aeration allows for controlling carbonate chemistry[a]	Not in agreement with corresponding G_{BW}
	Smaller changes in Ω_{arag} than AA method 3[a]	Aeration may not provide sufficient water motion
	Simple, affordable, and fast setup	Increased evaporation
AA method 3 (covered container with pump)	Pump provides continuous water motion	Carbonate chemistry not controlled, greatest changes in Ω^{a}_{arag}
	Agreement with corresponding G_{BW}	More complex and expensive setup
	No evaporation	

[a] See Table 2 for how much carbonate chemistry changed over the course of these incubations

are not maximized, the actual measurement mechanism of this technique is impeded (i.e., calcification may be occurring, but it cannot be measured in its full extent due to limited exchange of molecules across the DBL). Thus, a thicker DBL combined with limited exchange may have resulted in lower measured and/or real calcification rates in the incubations with airstones (AA method 2) compared to those using pumps (AA method 3) due to more limited water movement. Similarly, manual stirring every 5–10 min during the open-top incubations for the tropical corals (AA method 1) may have been less ideal than water motion provided by a pump and could thus have accounted for the slight tendency of G_{AA1} to underestimate G_{BW}, although the fact that they were not significantly different justifies manual stirring in this case. Therefore, despite the advantage of providing pCO_2 control and overall stabilizing carbonate chemistry (Table 2), incubations with aeration are not recommended, at least not for long incubations (Tables 1, 5).

Although water motion is certainly a critical factor during AA incubations, other factors such as changes in saturation state of the incubation water or nutrient release (Jacques and Pilson 1980) may also play an important role. For example, two studies that provided water motion via continuous magnetic stirring during AA incubations nevertheless found that G_{AA} were significantly lower than G_{BW} (Steller et al. 2007; Rodolfo-Metalpa et al. 2010). Rodolfo-Metalpa et al. (2010) showed that G_{AA} was significantly lower (up to 44%) than G_{BW} in the Mediterranean coral *Cladocora caespitosa*. However, they used different sets of corals for the two methods, much smaller incubation volumes (50 mL), and measured dark calcification rates during the day in darkness, all of which could have affected the observed calcification rates. Similarly, Steller et al. (2007) showed that G_{AA} of the coralline alga *Lithophyllum margaritae* was significantly lower than G_{BW} despite continuous magnetic stirring. However, drastic changes in saturation state of the incubation seawater may have resulted in artificially low G_{AA}. In their 10 °C treatment, A_T declined $\sim$100 μmol kg^{-1}, but in their 25 °C treatment, the decrease was more than 400 μmol kg^{-1} (a 20% reduction from the original value). Assuming a salinity of 35 ppt, an initial A_T of 2200 μmol kg^{-1} and constant pCO_2 of 385 μatm (open-flask setting to equilibrate with air CO_2), Ω_{arag} would have decreased by almost 1 unit (from 3.2 to 2.3) during the 60-h incubations. Drastic Ω reduction could thus have caused the apparent decline in G_{AA} and confounded their method comparison.

Ultimately, however, in a laboratory setting, the AA technique typically integrates calcification rates only over several hours or 2–3 d at best (Table 1), whereas the BW technique typically integrates over much longer timescales. Therefore, the assumption of constant G when extrapolating G_{AA} over longer timescales likely becomes increasingly flawed with increasing study duration. Given that the majority of studies compared G_{BW} and G_{AA} over many weeks to months (e.g., Holcomb et al. 2010; Rodolfo-Metalpa et al. 2010; Maier et al. 2013), this aspect may be at least as important as the incubation design. In our experiments, we allowed the corals to acclimate for several weeks and kept important environmental variables such as light, carbonate chemistry, and feeding constant, which likely promotes constant calcification rates over time. Furthermore, two out of three AA techniques resulted in G_{AA} that were not significantly different from G_{BW}, thus indicating that calcification rates were roughly constant over time despite incubations being conducted only once during the experiment.

Overall, we assessed a range of AA incubation protocols and found that two out of three protocols assessed here resulted in calcification rates that are comparable to BW-based rates. This strongly suggests that the two techniques are, in principle, comparable and highlights strengths and weaknesses of certain incubation designs (Table 5). However, since it was not planned a priori to combine the data from the two separate experiments for this method comparison, we were not able to compare these incubation designs using the same species, incubation chambers, incubation times, etc. Therefore, further study should be conducted to assess differences between general culturing and AA incubation setups as well as potentially variable calcification rates over time.

Critical factors in alkalinity depletion incubations and recommendations

This study highlights that AA incubations should be conducted in a stringent manner to produce calcification rates that are comparable to BW-based rates. Recommendations provided here are not intended to be an exhaustive list but rather to complement the detailed and useful guidelines of Riebesell et al. (2010).

Water motion is clearly one of the key factors for obtaining comparable calcification rates. The use of small pumps inside the incubation container gave the best results in this study and is recommended (Table 5). In principle, continuous magnetic stirring should also provide satisfactory water motion, though stir bar size and stirring speed should be optimized with regard to organism size and metabolism (e.g., Holcomb et al. 2014).

Another critical factor is the duration of alkalinity depletion incubations, which should be long enough to produce a good signal-to-noise ratio but short enough to prevent significant changes in carbonate chemistry that might, in turn, affect calcification rates. Clearly, this also depends on the volume of the incubation seawater relative to organism size and metabolism. Unfortunately, the potential influence of this factor is difficult to assess because many studies do not report the amount of alkalinity reduction over the course of their incubations. Riebesell et al. (2010) suggested that changes in A_T during incubations should be 3–10 times the analytical precision of the measurement and within 10% of starting A_T (or dissolved inorganic carbon). In this study, A_T reductions were well within this range in both experiments, and overall Ω_{arag} decreased by less than 0.33 units (Table 2). We are therefore confident that G_{AA} values reported in this work were not confounded by artificial changes in carbonate chemistry. Nevertheless, we stress the importance of measuring and reporting changes in A_T (and ideally also a second parameter of the carbonate system to assess changes in Ω_{arag}) over the course of alkalinity depletion incubations (including control incubations). This applies especially to long incubations that are conducted without pCO_2 control.

Finally, corrections for nutrient release should not be required for the majority of tropical coral species because nutrient release tends to cause negligible changes in A_T (Chisholm and Gattuso 1991), which is corroborated by the findings from this study. In temperate corals, this appears to be species-specific: nutrient release was found to be negligible in *C. caespitosa* (Gazeau et al. 2015) but significant in *Astrangia* spp. (Jacques and Pilson 1980), though the results from our study suggest that nutrient release varies from study to study (otherwise, G_{AA3} should not have been in good agreement with G_{BW}). Therefore, tests should be performed to assess nutrient release for each study species.

In summary, we found that the buoyant weight and alkalinity anomaly techniques produce comparable estimates of coral calcification rates under various pCO_2, temperature, and nutrient conditions but that the similarity of estimates depends on how the alkalinity depletion incubations are conducted (Table 5). This suggests that weaknesses in incubation protocols rather than an incompatibility of the two techniques resulted in the higher sensitivity of G_{AA} to ocean acidification compared to G_{BW} reported by Chan and Connolly (2013). Since each technique has inherent advantages and disadvantages, the two methods should ideally be used in a complementary manner where researchers take advantage of the AA technique to compare day and night calcification and to measure community calcification rates, while using the BW technique to measure integrated calcification rates over longer time periods or when resources are limited.

Acknowledgments We thank all staff at Reef Systems Coral Farm for logistical support. We thank E. Zebrowski, M. Berzelis, M. Ringwald, S. Levas, and S. Blackhurst at The Ohio State University for their assistance in the field and in the laboratory. We thank Jimmie (Luke) Snaric for carrying out the CO_2 degassing experiment at Texas A&M University Corpus Christi. This work was supported by funding from the US National Science Foundation (NSF-EF-1041124, 1040940, 1041070 to AGG, MEW, and WJC, respectively). QL and HX acknowledge the partial support of their respective home institutions for their visit of the Cai laboratory at the University of Georgia when part of this work was accomplished. XH was a postdoctoral researcher in WJC's laboratory at the University of Georgia during the experimental portion of this work, and also acknowledges the startup support provided by the College of Science and Engineering, Texas A&M University—Corpus Christi during the preparation of this manuscript. At WHOI, this work benefited from the assistance of B. Belastock, M. Brosnahan, N. Cantin, A. Cohen, G. Gaetani, S. Gallager, P. Henderson, K. Hoering, F. Keller, D. McCorkle, C. McGraw, J. Ries, T. Rioux, N. Shimizu, R. Schenk, J. Smith, A. Tarrant, K. Thompson, N. Trowbridge, A. Wang, D. Wellwood, M. White, and A. York. This material is based upon work supported under a US National Science Foundation Graduate Research Fellowship and International Society for Reef Studies/Ocean Conservancy Fellowship awarded to MH, and an NSF Grant (Oce-1041106) awarded to Anne Cohen.

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