Measuring coral calcification under ocean acidification: methodological considerations (#18120)

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Measuring coral calcification under ocean acidification: methodological considerations

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As the oceans become less alkaline due to rising CO_2 levels, deleterious consequences are expected for calcifying corals. Predicting how coral calcification processes will be affected by on-going ocean acidification (OA) requires an accurate assessment of absolute calcification values as well as an understanding of the relative importance that decreasing calcification and/or increasing dissolution play for the overall calcification budget of individual corals. Here, we assessed the compatibility of the ^{45}Ca-uptake and total alkalinity (TA) anomaly techniques, typically used as measures of gross and net calcification, respectively, to assess coral calcification at pH_T 8.1 and 7.5. Considering the differing buffering capacity of seawater at both pH values, we were also interested to what extent coral calcification alters the seawater carbonate chemistry under prolonged incubation in sealed chambers, potentially interfering with physiological functioning. Especially at reduced pH, substantial changes in carbonate system parameters for incubation times beyond two hours in our experiment demonstrate the necessity to test and optimize experimental incubation setups when measuring coral calcification in closed systems under OA conditions. Our data indicate that while calcification estimates by both techniques are very similar under ambient conditions, they disagree at reduced pH, where net calcification estimates by TA are erroneously higher than gross calcification estimates by ^{45}Ca. Considering also previously published data, we show that the discrepancy between both techniques becomes smaller for larger absolute calcification values. With regard to the assessment of coral dissolution based on the calculated difference between net and gross calcification, we advocate an alternative approach.

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Abstract

As the oceans become less alkaline due to rising CO_2 levels, deleterious consequences are expected for calcifying corals. Predicting how coral calcification processes will be affected by on-going ocean acidification (OA) requires an accurate assessment of absolute calcification values as well as an understanding of the relative importance that decreasing calcification and/or increasing dissolution play for the overall calcification budget of individual corals. Here, we assessed the compatibility of the ^{45}Ca-uptake and total alkalinity (TA) anomaly techniques, typically used as measures of gross and net calcification, respectively, to assess coral calcification at pH_T 8.1 and 7.5. Considering the differing buffering capacity of seawater at both pH values, we were also interested to what extent coral calcification alters the seawater carbonate chemistry under prolonged incubation in sealed chambers, potentially interfering with physiological functioning. Especially at reduced pH, substantial changes in carbonate system parameters for incubation times beyond two hours in our experiment demonstrate the necessity to test and optimize experimental incubation setups when measuring coral calcification in closed systems under OA conditions. Our data indicate that while calcification estimates by both techniques are very similar under ambient conditions, they disagree at reduced pH, where net calcification estimates by TA are erroneously higher than gross calcification estimates by ^{45}Ca. Considering also previously published data, we show that the discrepancy between both techniques becomes smaller for larger absolute calcification values. With regard to the assessment of coral dissolution based on the calculated difference between net and gross calcification, we advocate an alternative approach.

Introduction

The continuing increase in atmospheric CO_2-concentration has led to measurable changes in the carbonate chemistry of the oceanic system, summarized under the term ocean acidification (OA; Kleypas *et al.*, 2006). These changes involve an increase in total dissolved inorganic carbon (DIC) and a shift in the carbon equilibrium towards pCO_2, which lead to a reduction in overall aragonite saturation state (Ω_{arag}) and pH (Kleypas *et al.*, 1999, Zeebe & Wolf-Gladrow, 2001). Current worst case climate models project a further decrease of surface seawater pH by 0.3-0.4 pH units until the end of the 21st century (IPCC, 2013). These continuing shifts in seawater pH and aragonite saturation state affect many marine organisms that form biogenic aragonite; among them scleractinian reef-building corals.

Coral calcification is the fundamental biological process that provides the physical three-dimensional platform for the existence of coral reef communities. However, deposited skeleton in a reef is subject to various forms of physical, chemical and biologically-mediated erosion, which causes the dissolution of reef sediments and skeleton. This dissolution is part of the natural turnover of matter in the reef community and can offset 20-30% of reef calcification (Barnes, 1988, Silverman *et al.*, 2007). It is widely accepted that OA will increase overall dissolution of coral reef communities (e.g. Andersson *et al.*, 2011, Eyre *et al.*, 2014, Silverman *et al.*, 2009) and some projections predict that many of them will even go from net accretion of reef skeleton to net dissolution by mid-century ($pCO2$ ~560 ppm; Silverman et al., 2009).

With scleractinian corals as major reef builders, a large body of research has thus been dedicated to the performance of individual coral species under varying degrees of OA. While

studies generally demonstrate a decline in net calcification (NC) to varying degrees, a few show also insensitivity of some species to OA conditions (reviewed in Table S1; Chan & Connolly, 2013). The variety in the calcification-dependent growth response to low seawater pH may reflect the true variability/plasticity in the ability of some corals to maintain calcification rates over a broad range of seawater Ω_{arag}. On the other hand, there is also a high degree of methodological variation in design, choice of pH lowering agents, interaction of one or multiple environmental factors along with pH (e.g. temperature, light), animal acclimation period, experimental incubation period, and employed techniques to determine calcification rates that contribute to the observed variability. Recognizing the fundamental impact that OA has on calcifying organisms, there is now a renewed interest for calcification studies of corals.

While the occurrence of net dissolution under OA conditions and during the night has been observed for some species, the contribution of skeleton dissolution (gross dissolution; GD) to changes in NC remains unknown. Commonly, it is assumed that changes of NC can be the result of a decrease in gross calcification (GC, the calcification rate before any deductions for dissolution), increased GD, or a combination of both following the equation NC = GC + |GD| (Fig. 1; Andersson & Mackenzie, 2012, Comeau *et al.*, 2014, Langdon *et al.*, 2010). However, this conceptual relationship has never been experimentally validated to see to what extent is possible to quantify dissolution from measurements of NC and GC.

The most common methods that are widely used to measure NC in living corals are the "buoyant weight" and "total alkalinity" technique (Table 1; Langdon et al., 2010). While the total alkalinity (TA) technique is based on tracking changes in TA in the incubation medium (Smith &

Key, 1975, Chisholm & Gattuso, 1991), the buoyant weight (BW) technique is a direct measure of colony growth. The TA is measured by acidimetric titration of the total inorganic carbon in the seawater sample. From the TA equation one can infer the calcification and dissolution of $CaCO_3$: precipitation of 1 mole $CaCO_3$ leads to a 2 moles decrease in TA, whereas dissolution causes the reverse effect (Wolf-Gladrow *et al.*, 2007). While these two methods are generally considered to give estimates of NC and provide comparable calcification rates (Schoepf *et al.*, 2017), the only method likely to provide measurements of gross calcification (GC) over short incubation periods is the ^{45}Ca-labelling technique. GC is commonly determined by direct incorporation of the radio-isotope ^{45}Ca into the skeleton using fully covered microcolonies (Goreau, 1959, Goreau & Goreau, 1960, Tambutté *et al.*, 1995). The procedure involves incubation in ^{45}Ca-labeled seawater, dissolving the skeleton in acid and measuring the incorporated radioactivity with a liquid scintillation counter. The TA and ^{45}Ca techniques are the only ones considered to have sufficient resolution to detect small changes in calcification over short-term incubations (Chisholm & Gattuso, 1991, Langdon et al., 2010) and to our knowledge, Smith and Roth (in Smith & Kinsey, 1978) and (Tambutté et al., 1995) are the only studies to date to provide a direct comparison of the TA and ^{45}Ca techniques for corals for ambient pH conditions. The accurateness and compatibility of both methods for incubations under reduced pH conditions has not yet been experimentally tested.

In the present study, these two methods for estimating NC and GC were compared on long-term acclimated (16 months) *Stylophora pistillata* microcolonies under ambient and reduced pH in order to test their compatibility in providing consistent calcification estimates ~~even~~ in the

context of OA research. Since TA calcification measurements are commonly combined with respiration measurements using closed (sealed) vessels, it was furthermore tested to what degree incubation time affects the stability of carbonate system parameters and the calcification rates, because especially at reduced pH, biological processes might amplify changes in these parameters due to the reduced buffering capacity of seawater.

Materials and procedures

Coral preparation and acclimation to pH treatment

Stylophora pistillata was collected by scuba diving from a depth of 4-8 m near the Interuniversity Institute for Marine Sciences (IUI) in the Red Sea, Eilat, Israel (29°30'N 34°55'E), in an area supervised by the Israel Nature and Parks Authority. Only a single colony was used for the calcification measurements in order to remove the effect of genetic variability for the tested treatments. The colony was fragmented into 36 fragments and these were suspended on nylon threads in flow-through water tables under a constant temperature of 25°±0.5C, regulated by an array of 300W BluClima aquarium heaters (Ferplast Spa, Vicenza, Italy) in an air-conditioned room. This setting allowed the tissue to grow over the exposed skeleton, creating microcolonies (definition sensu Almoghrabi *et al.*, 1993). All pieces were checked to be free of boring organisms. Light was provided by a metal halide lamp (14,000 K, 400W/D, Osram GmBH, Germany) and photosynthetically active radiation (PAR) measured as ~170 μmol photons m^{-2} s^{-1} (10h L:14h D photoperiod; quantum irradiance meter LiCor, Lincoln, Nebraska, USA). All microcolonies were exposed to two pH_T levels (pH in total scale): 8.09±0.01 (pCO_2 = 387 μatm;

present day ambient pH) and 7.49±0.20 (pCO_2 = 1917 µatm**;** predicted pCO_2 level for 2300 with Ω_{arag} close to 1, Table 2, Caldeira and Wickett 2005) and maintained under these conditions for over 16 months (thus, relative to most other studies acclimation time was very long, cf. Table 1, S1). pH was tightly regulated and monitored over the acclimation period (see below). Corals were fed each week with freshly hatched *Artemia* sp. nauplii and a mixture of crushed fish.

Control of seawater pCO_2

Seawater was pumped from a depth of 30 m into 1000 L tanks, where the pH was manipulated to reach a fixed value. The incoming seawater presented a very stable chemistry during the whole experiment period: salinity of 40 ± 0.2 ‰, pH_T of 8.09 ± 0.017, total alkalinity of 2505 ± 0.005 µeq kg^{-1}, as reported by the Israel National Monitoring Program (NMP) of the Gulf of Eilat (NMP, 2016). A pH electrode (S-200C, Sensorex, CA, USA) located in each water table was connected to a pH controller (Aquastar, IKS ComputerSysteme GmbH, Karlsbad, Germany), monitoring the pH (software Timo, Matuta, Germany) and adjusting the bubbling of CO_2 (from a CO_2 cylinder) accordingly. Well-mixed water from each tank flowed continuously into the corresponding section (150 L) of the water table and pH was monitored daily. Seawater samples from the pH system were taken 8 times over the acclimation period to monitor TA (2491±9 and 2501±6 µeq kg^{-1} for pH_T 7.5 and 8.1, respectively; Table 2). All pH values given in this paper refer to the pH_T scale.

Incubation procedure for measurements of calcification

After 16 months of pH acclimation in water tables, microcolonies (2.5-3.0 cm in length, N=6 per treatment and time point) were incubated in sealed (no gas exchange; no headspace) incubation vessels (40 mL Septa vials with additional paraffin wrap, clear borosilicate glass; 2.5x10 cm; Thomas Scientific, Inc, Swedesboro, NJ, USA) containing 40 mL filtered seawater (FSW; 0.45 μm) at the two pH levels for 2h, 4h, and 6h. Temperature and light intensity during the incubation were similar to those provided during the preceding 16 months of acclimation (see above), while an orbital shaker provided constant water motion. Applicability of the shaker in contrast to conventionally used stir bars was tested in a preliminary experiment (Fig. S1). Prior to the incubation, the colony surface and attached nylon strings were carefully cleaned of epiphytes and algae. Microcolonies in the incubation vials were hanging without any wall contact by using a T-shaped string (Fig. S2). The TA and ^{45}Ca experiments were carried out over two consecutive days, using the same microcolonies and time of day to avoid effects of diurnal variations in physiology (Edmunds & Spencer-Davies, 1988). After TA measurements, the respective microcolonies were returned to the water table overnight and used for ^{45}Ca measurements the following day. This allowed minimizing physiological differences due to handling or cumulative stress between both measurements. The chosen repeated measures design clearly represents a practical compromise. Although the dual measurement of the ^{45}Ca-uptake into the skeleton and the TA change in the surrounding seawater would have been preferable, the subsequent alkalinity measurement of radioactive seawater with a radioactive contamination of the titrator and the associated equipment was not justifiable for reasons of lab safety (note however that such an

159 approach was taken by Tambutté et al. 1995). While day-to-day variations in calcification cannot
160 be ruled out with certainty, we found no significant statistical effect of this in the complete
161 dataset (i.e. paired response in separate 2h, 4h, 6h incubations).

162 *Total alkalinity technique*

163 Net calcification rates of corals were determined using the TA anomaly technique
164 (Chisholm & Gattuso, 1991). Water samples were collected for TA at the beginning and end of
165 the incubation from each treatment to determine carbonate chemistry and stored until analysis in
166 sealed, bubble-free brown glass vials (borosilicate glass) in the dark at 4°C. Calcification rates
167 were calculated from the difference between TA measured at the beginning and the end of each
168 incubation period (ΔTA; corrected for blank values from filtered seawater only incubations with
169 N=3), according to the equation by (Schneider & Erez, 2006). Rates were normalized to skeleton
170 dry weight:

171
$$\text{Calcification } (\mu\text{mol CaCO3 h - 1 g - 1 dry skeleton}) = \frac{\frac{\Delta TA}{2} \times (\text{Vvessel - Vcoral}) \times 1000 \times 1.028}{W \times T}$$

172 ΔTA (in μeq kg^{-1}); V_{vessel} is the volume of the experimental vessel (in mL); V_{coral} is the
173 displacement volume of the coral (in mL); 1.028 is the density of seawater in the northern Gulf of
174 Eilat (in g mL^{-1}); W is skeleton dry weight (in g) and T is the incubation duration (in hours).

175 *^{45}Ca technique*

176 Measurements of GC were based on the improved ^{45}Ca protocol (Tambutté et al., 1995).
177 Microcolonies were placed in filtered seawater (FSW; 0.45µm) with a total activity of 360 kBq
178 (as $^{45}Cl_2$, 1958.18 MBq mL^{-1}, Perkin-Elmer Life and Analytical Sciences). Dead microcolonies,
179 killed with 2% formaldehyde, were included in the experiment as a control for isotopic exchange
180 (Al-Horani *et al.*, 2005). To determine the specific activity, 100 µL aliquots were taken at the
181 beginning and end of each incubation period. Three incubation vessels were left without
182 microcolonies to serve as a seawater-only control. Following the labelling period, specimens
183 were immersed in 600 mL FSW for 1 min, and then rinsed (5 x 1 min) with 10 mL of ice-cold
184 glycine-high calcium medium (50 mM $CaCl_2$, 950 mM Glycine, pH 8.2). Labelled specimens
185 were then incubated for 30 min in vessels containing 20 mL of FSW. Water motion was provided
186 by a shaker. Following efflux incubation, microcolony tissue was removed using 2 M NaOH for
187 20 min at 90°C. Following tissue hydrolysis, the skeleton was first rinsed with 1 mL NaOH
188 (Houlbrèque *et al.*, 2003), then thoroughly rinsed with FSW, followed by double distilled water.
189 The solution from the first rinse was added to the tissue hydrolysate, while the remaining rinsing
190 solutions were discarded. Finally, skeletons were dried at 70°C, weighed, and subsequently
191 dissolved in 12 M HCl. Samples (500 µL) of skeleton digest or tissue hydrolysate were added to
192 10 mL Ultima Gold AB (PerkinElmer) scintillation liquid and measured on a scintillation counter
193 (Tri-carb 1600TR, Packard). Sample count rates (CPM, total counts per minute) were obtain
194 several times over 2 days and the average difference between readings was 0.8±0.54%. Total

195 calcification was then calculated from the activity recorded in the skeleton digest and seawater
196 control samples and normalized as μmol $CaCO_3$ per skeleton dry weight using the formula:

197 $$\text{Calcification (μmol CaCO3 h - 1 g - 1 dry skeleton)} = \frac{(\text{Activitysample} \times \frac{1.17}{\text{Activityseawater}})}{W \times T}$$

198 Where $Activity_{sample}$ is the total counts per minute (CPM) in the dissolved skeleton sample;
199 $Activity_{seawater}$ is the total CPM in 100 μL seawater sample (control); *1.17* is the amount of Ca^{2+} in
200 100 μL ambient seawater (in μmol); *W* is skeleton dry weight (in g) and T is the incubation
201 duration (in hours). The amount of ^{45}Ca uptake by dead specimens (covered with tissue) was
202 subtracted from the amount measured in intact (live) specimens.

203 *Calculation of seawater carbonate system*

204 Value of TA of accurately weighed seawater samples (12.3 g) were measured to the
205 second end point (Almgren *et al.*, 1983) using an automatic potentiometric titration (Mettler-
206 Toledo GmbH, DL67 titrator; resolution of burette volume: 1/5000). The TA was determined in
207 triplicate and computed using the Gran equation (DOE, 1994) with pH values lower than 3.9 for
208 creating the Gran plot. Accuracy of TA analysis was checked against certified seawater reference
209 material prepared by Andrew Dickson (Scripps Institute of Oceanography). The differences
210 between triplicate samples were less than 6 μeq kg^{-1}. The pH electrodes (Mettler-Toledo DG-
211 111–SC; Stockholm, Sweden) were calibrated daily before using the titrator (The manufacturer's
212 technical specifications are ±0.03 pH for both precision and accuracy). The acid concentration
213 was 0.049 N HCl (JT Baker, Phillipsburg, NJ). In the series of experiments that compared gross

and net calcification, a new titrator was utilized: a Metrohm 862 compact Titrosampler that uses at least 35 g seawater samples (autosampler combined with titrator; potentiometric measuring accuracy of ±0.003 with resolution of 0.001; resolution of burette volume: 1/10000). Hence, experimental samples containing only 40 mL, were diluted by a factor of 3 (to have enough water for triplicate measurements of TA) and the acid concentration was set to 0.025 M. TA was calculated using the first derivative of the curve for the evaluation of the exact end point. Prior to measurements, water samples were filtered (0.22 µm EMD Millipore Millex sterile syringe filters). Water samples for analysis were stored in darkness at 4°C in brown glass bottles, filled up to the top with a gas tight screw, and processed within two weeks of collection.

pH measurements were carried out using a CyberScan pH meter (pH/Ion 510 Eutech Instruments with automatic temperature compensation) and CyberScan gel-filled pH combination electrode. Prior to experiments, the pH electrode was calibrated against National Bureau of Standards (NBS) scale buffers of 4.01, 7.00 and 10.00 (Mettler Toledo)at 25°C and was soaked in seawater for at least 1h before measurement. The manufacturer's technical specifications of the pH meter were 0.01 pH for resolution and ±0.01 (standard error) for accuracy.

Components of the carbonate system (pCO_2, CO_3^{2-}, HCO_3^-, DIC concentrations and Ω_{arag}) were calculated from TA along with pH values, temperature and salinity using the CO2SYS program, version 1.03 (Lewis *et al.*, 1998, Pierrot *et al.*, 2006). The pH_{NBS} were shifted onto the total pH scale (pH_T) by subtracting -0.11 (Zeebe & Wolf-Gladrow, 2001), which includes a minor correction for [SO_4^{2-}] and the stability constant of HSO_4^- at a salinity of 40.7‰. The thermodynamic carbonate dissociation constants for activity scales (K1 = 5.845±0.008 and K2 =

8.945±0.013) were attained from (Mehrbach *et al.*, 1973) and the refit from (Dickson & Millero, 1987). The input parameters for pressure (10 dbar), total P (0.03 µmol kg^{-1}) and total Si (0.68 µmol kg^{-1}) were obtained from the NMP of the Gulf of Eilat (NMP, 2016).

Data normalization

For accurate determination of TA changes in the seawater volume, coral volume was determined by measuring its displacement weight in seawater following Archimedes' principle and assuming an approximate density of 1.028 g cm^{-3} for Red Sea seawater. Normalized calcification rates were obtained by determining skeleton dry weight, using a vibra balance (Shinko Denshi Co., Ltd., Japan; accuracy ~1 mg).

Statistical analysis

Due to the paired nature of the measurements, the overall dataset was analysed with a repeated measures ANOVA. This way, the within subjects effects allow to test for consistent differences in calcification rates between both estimation methods (TA *vs*. ^{45}Ca) as a function of incubation time (2h, 4h, 6h), seawater pH_T (8.1 *vs*. 7.5) and the combination of both, whereas the between subject effects test whether overall calcification rates differ between pH and incubation time. All calcification rates were cube root transformed to achieve normality as tested by the Shapiro-Wilk test. Differences between estimation methods due to seawater pH were also tested for the 2h time point, using the overall fitted model (repeated measures ANOVA as above) as well as a paired t-test for each pH.

The relationship between absolute estimates of calcification (in nmol) between TA and ^{45}Ca was evaluated using geometric regression fitting and by directly comparing $^{45}Ca/Ta$ ratios over a range of calcification values. Geometric regression was preferred over linear regression since no causality can be established between both variables and both have an inherent measurement error. Note that geometric regression equations still depend on the assignment of x and y. To test whether the relationship between both methods is affected by seawater pH, $^{45}Ca/TA$-ratios from both treatments after 2h were tested with a t-test. *Post hoc* power analysis was conducted for all significant main and interaction terms. All statistical analyses were performed in JMP 11.2.1 (SAS Institute, Cary, NC, USA).

For comparison with literature data (Smith & Kinsey, 1978, Tambutté et al., 1995), the software WebPlotDigitizer (Rohatgi, 2015) was employed to extract the data points from the original publication graphs, since the raw data is no longer available (S. Smith, E. Tambutté personal communication). The extracted data is provided as supplementary information for future reference (Table S2).

Results and discussion

Changes in seawater chemistry

Substantial changes in the carbonate chemistry (TA, DIC, pH, pCO_2, HCO_3^-, CO_3^{2-} and Ω_{arag}) were recorded inside the incubation vessels during the time course of the experiment at both pH treatments (Table 3, S3). A general decline in DIC was correlated with an increase in seawater pH and a shift within the carbon equilibrium. For an incubation period beyond two

hours, a substantial pH shift (+0.4-0.9 units) with $CO_{2(aq)}$ depletion of more than 70%, as well as considerable changes (≥10%) in seawater DIC were observed irrespective of pH (Table 3).

At constant temperature and salinity, alterations in carbonate system parameters such as pH and TA are driven mainly by biological activities of the coral holobiont such as photosynthesis, respiration, and calcification (Schulz & Riebesell, 2013). Photosynthesis and calcification decrease the seawater DIC, while respiration and dissolution increase the DIC. However, only calcification and dissolution alter the TA (Zeebe & Wolf-Gladrow, 2001). The photosynthetic activity of the dinoflagellate symbiont is the main driver for the observed changes in total DIC and DIC speciation (with subsequent pH shift) as CO_2 is removed and fixed in biomass at a much higher rate than HCO_3^-/CO_3^{2-} incorporation into the skeleton. These shifts were significantly higher in the reduced pH treatment compared to ambient pH likely due to the lower buffering capacity of CO_2-enriched seawater, specifically at pH_T 7.5, where seawater reaches its minimum buffering capacity (Delille *et al.*, 2005, Egleston *et al.*, 2010, Riebesell *et al.*, 2007, Suzuki, 1998). Near pH_T 7.5, where DIC~TA, any changes in DIC and TA will cause a similar magnitude change in $[CO_2]$, $[H^+]$, and Ω and a sharp change in pH (Egleston et al., 2010).

Best practice guidelines recommended changes in TA should be at least 3- to 10-fold (Langdon et al., 2010) the analytical precision of the instrument (e.g. ΔTA ~6-20 µmol kg^{-1} seawater), but these changes along with changes in DIC should not exceed 3% (Schulz *et al.*, 2009) or 10% (Langdon et al., 2010) of the absolute values. Given the observed stronger changes in seawater chemistry at lower pH over time in our experiment, one can see that the trade-off between using a small incubation volume to accurately measure changes in TA versus using a

large volume to minimize changes in carbonate chemistry becomes a crucial issue for measurements under OA conditions, especially in closed systems. For calcification measurements with symbiotic corals at reduced pH in closed vessels, incubation time must be minimized as long as the seawater carbonate parameters are not kept constant. In addition, increasing oxygen saturation in closed vessels due to photosynthesis is also an important factor to consider when measuring zooxanthellate corals in the light. In a similar experiment, very high pO_2 levels (200-250%) were recorded after 4h and 6h of incubation, at both pH treatments (data not shown). Hyperoxic conditions promote the generation and accumulation of reactive oxygen species (Gerschman *et al.*, 1954) with potentially negative effects that interfere with the normal physiological performance and the subsequent estimate of calcification rate.

The problem of fundamental changes in seawater chemistry and their negative feedback for coral physiology can essentially be captured in the ratio of coral biomass to chamber volume and incubation time. The presented experimental data clearly confirm that these two factors define the applicability and limits of closed chamber incubations as highlighted previously, but become especially important in the context of measurements under OA conditions.(Chisholm & Gattuso, 1991, Langdon & Atkinson, 2005, Langdon et al., 2010, Schulz et al., 2009). Note that coral size rather than coral surface area is used in the following, since surface area as a size proxy was not always available from the discussed studies. In the present experiment, the ratio between incubation volume (mL) and coral size (cm) was 13-16 (corresponding V_{vessel}/V_{coral}-ratios ranged from 20 to 44) and incubations beyond 2h already caused large shifts in carbonate parameters and pO_2. This should be a general point of consideration, since previous studies that used the same

316 coral species, similar conditions of temperature and light intensity (~25°C; ~150-170 µmol
317 photons m^{-2} s^{-1}), and used similar (10-17; (Furla *et al.*, 2000) or even smaller (6-8; (Tambutté et
318 al., 1995)) incubation volume/coral size-ratios, incubated corals for up to 3h (incubation volumes:
319 7-10 mL), thus most likely experiencing similar shifts in the carbonate species and/or O_2
320 concentration. The other extreme, employing very large incubation volumes relative to coral size,
321 bears the risk of approaching the detection limit of the TA titration method, even with longer
322 incubation times. For example, ΔTA over 2h approached less than three times the analytical
323 precision for most of the treatments for a volume/size ratio of 225 in the study of (Takahashi &
324 Kurihara, 2013). It should be favourably noted that the studies that experienced this issue and
325 used alkalinity changes as low as 3 µmol kg^{-1} to calculate calcification rates (Hossain & Ohde,
326 2006, Ohde & Hossain, 2004, Takahashi & Kurihara, 2013) belong to the few publications that
327 explicitly report the measured changes in the carbonic acid system in the incubation vessels. Our
328 experimental data reaffirm the necessity of reporting changes in the carbonic-acid system
329 parameters not only for pre-experimental pH acclimation, but also for actual incubation
330 experiments that yield calcification data, following previously suggested best practice guidelines
331 (Andersson & Mackenzie, 2012, Langdon et al., 2010). The need for a valid description of the
332 whole carbonate system is further emphasised by the study of (Hoppe *et al.*, 2012) on the
333 uncertainties in the calculated carbonate chemistry when using only two measured parameters.
334 Considering the varying performance in calcification, photosynthesis, and respiration between
335 coral species and environmental conditions (e.g. pH, light, temperature), preliminary experiments

336 can be beneficial in determining minimal vessel volumes, dependent on coral size for the
337 respective incubation time in closed systems.

338 *Effect of incubation time and estimation method on derived calcification rate*

339 During the 16 months of acclimation at both pH treatments all coral microcolonies
340 survived and remained visibly healthy (extended polyps; no bleaching). Considering data from all
341 incubation periods, calcification rate estimates from both techniques were not significantly
342 different, nor were there consistent differences between both techniques related to pH or
343 incubation time (Fig. 2, Table 4). Overall, calcification rates were significantly higher at reduced
344 pH with no significant effect of incubation time (Fig. 2), but these findings are not of biological
345 significance since only a single individual was tested.

346 Considering only the calcification rates obtained from 2h of incubation as physiologically
347 useful data (see earlier discussion) shows that absolute estimates of calcification differed between
348 both methods depending on pH (Fig. 2, Table 4). Whereas no significant difference was found at
349 ambient pH (pairwise One-way ANOVA $F_{1,10} = 0.472$, p = 0.5228; N=6), calcification estimates
350 at pH_T 7.5 were surprisingly higher for the TA technique compared to ^{45}Ca method ($F_{1,10}$ =
351 14.947, p = 0.0118*, N=6). The implications of this finding, including previously published
352 experimental date, will be discussed in the following paragraph.

353 *Can dissolution simply be derived from the difference between net and gross calcification?*

354 Net calcification rates can be altered as result of changes in dissolution and/or gross
355 calcification (Fig. 1). Distinguishing true changes in gross calcification (as result of biological

356 impairment) from changes in gross dissolution rates is therefore of importance to assess the real
357 impact of pH stress. Paradoxically, the few studies that measure GC and NC that would allow an
358 estimate for dissolution found higher rates for NC in comparison to GC (Rodolfo-Metalpa *et al.*,
359 2015, Tambutté et al., 1995), with the exception of Smith and Kinsey (1978). Some techniques
360 might not be suitable at all for such calculations, as indicated by a 7- to 11-fold overestimation of
361 calculated GC, when using rates of NC and dissolution from the buoyant weight technique and
362 comparing it with ^{45}Ca GC rates (Rodolfo-Metalpa et al., 2015). In this case, the raw data even
363 suggested that buoyant weight-based dissolution rates in the cold-water coral *Desmophyllum*
364 *dianthus* at pH 7.7 were larger than actual ^{45}Ca GC rates, despite measurable NC (as calculated
365 from provided raw data; Rodolfo-Metalpa, personal communication). Correlating blank-corrected
366 calcification values from the TA and ^{45}Ca technique from the 2h dataset at both pH treatments
367 confirms the strong significant positive correlation between both techniques (Fig. 3A), consistent
368 with previous findings (Smith & Kinsey, 1978, Tambutté et al., 1995). Nevertheless, it still
369 suggests that TA overestimates calcification values relative to the ^{45}Ca technique, because
370 ^{45}Ca/TA ratios tend to be below 1 (Fig. 3B).

371 Previous work by Smith and Roth (in Smith and Kinsey 1978) incubated tips of *Acropora*
372 *formosa* for 30 min in test tubes containing ^{45}Ca-labelled seawater, whereas Tambutte et al.
373 (1995) improved this ^{45}Ca protocol and labelled microcolonies of *S. pistillata* in seawater for up
374 to 3h. Both studies confirmed a strong correlation (r=0.95-0.99) between the TA and ^{45}Ca
375 estimates, but found opposing results with regard to absolutes calcification estimates between
376 these two methods. While the slope of the geometric regression was similar between both studies

and similar to the obtained values here (Fig. 3A), the regression and its extrapolated intercepts relative to the 1:1 line suggested opposite findings: 1) the presence of ^{45}Ca in the skeleton even without measurable changes in TA (Smith and Kinsey 1978; Fig. 3A, B) and 2) changes in TA without detectable ^{45}Ca incorporation in the skeleton (Tambutte et al. 1995; Fig. 3A, B). Physical adsorption of ^{45}Ca to the exposed skeleton surface at the fracture site, rather than biologically mediated incorporation, was suggested as the source for the overestimation by the ^{45}Ca method by Smith and Kinsey (1978). The use of completely tissue-covered microcolonies was able to minimize this effect in the study of Tambutte et al. (1995) and the resulting negative intercept was interpreted as loss of radioactivity during washing steps or general time lag due to the ^{45}Ca loading of extracellular and tissue compartments. Since both studies chose an overall line-fitting method to compare TA and ^{45}Ca estimates instead of comparing the values directly, it was not apparent that the absolute level of over- or underestimation of either method was not constant, but largely dependent on the absolute amount of deposited $CaCO_3$ (Fig. 3B). This is expected given that the relative overestimation of ^{45}Ca adsorption at exposed skeletal parts, or underestimation due to ^{45}Ca loss, should represent a small, constant value that is related to surface and porosity of the skeleton and is not proportional to the amount of deposited $CaCO_3$. Thus, the influence of such artefacts on the ^{45}Ca/TA relationship becomes less important for larger calcification values (see Fig 3B). We confirmed that the ^{45}Ca isotopic equilibration of dead, covered skeletons (used as blanks) was rather constant after 2h and 6h, with 0.14±0.03 and 0.19±0.02 µmol $CaCO_3$ g^{-1} dry skeleton (mean±SD; N=3), respectively. Figure 3B illustrates that for a sufficiently high $CaCO_3$ deposition (>1500 nmol from TA estimate), the ^{45}Ca/TA ratio

approaches a rather constant value with 1.19±0.02 (mean±SE, N=3, Smith and Kinsey 1978), 0.77±0.01 (N=10; Tambutte et al. 1995), 0.94±0.03 (N=5; this study pH_T 8.1), and 0.81±0.02 (N=5; this study pH_T 7.5). Thus, when interpreting the relationship between these two methods one must consider two important aspects: (1) the correlational strength, which demonstrates the linear relationship between both methods over a range of values, and (2) the numerical ratio itself, which represents the difference in the absolute calcification estimate between both methods and is assumed to provide an estimate for the occurrence of dissolution. For the first aspect, we conclude that both methods correlate well in both pH treatments. For the second point however, we have to conclude that the apparent tendency for GC/NC ratios to be <1 in our study and the study of Tambutte et al. (1995) contradicts the generally assumed relationship of NC ≤ GC. This is furthermore problematic since TA estimates in our experiment are factually a measure of GC, because we demonstrated in a separate experiment, that dissolution (if occurring) was not detectable. When testing bare skeletons of similar size in the same vials over a slightly longer period of time (3h) and even lower pH (pH 8.1, 7.6, and 7.3 with N=9 each), we found that 25 of the obtained 27 ΔTA values were less than 18 μeq kg^{-1} and therefore below the recommended detection limit of at least three times the precision of the used titrator (Table S4). Thus, for the case of no detectable dissolution, both methods would represent measures of GC, but still provide different estimates for absolute calcification, especially at reduced pH. Even if dissolution occurred under reduced pH conditions, the ^{45}Ca/TA ratio shifted in the wrong direction, erroneously indicating that NC increases relative to GC. This violates the assumed relationship of NC = GC + |GD| and raises questions whether a comparison of these two sensitive methods is

419 really appropriate to provide accurate values for GD under OA conditions. Limited by sensitivity
420 of common methods, assessing gross dissolution and its relation to net calcification
421 simultaneously is clearly a challenge.

422 **Conclusion**

423 Stating that reduced pH decreases coral calcification based on measurements of net calcification
424 alone fails to identify whether there is a reduced biological capability to form a new skeleton or
425 whether skeletal dissolution outweighs the biologically-mediated deposition (see Fig. 1). We
426 demonstrated the significance of potential changes in TA and DIC speciation as a function of
427 incubation time when using photosynthetically active corals for two common techniques that
428 provide estimates for NC and GC. Given the remaining uncertainties about deriving gross
429 dissolution from different NC-GC comparisons (BW/TA vs. ^{45}Ca), further experiments are
430 clearly required to provide an explicit value for gross dissolution of skeletal material in living
431 corals. One experimental approach that was previously suggested, but to our knowledge has not
432 yet been experimentally tested, is to incubate a coral in ^{45}Ca-spiked seawater for a few hours/days
433 in order to completely label the skeleton so that a subsequent incubation in seawater would allow
434 the direct assessment of ^{45}Ca-dissolution from the skeleton (after correcting for the isotopic
435 equilibration) (Langdon et al., 2010). Simultaneous incubation with a different detectable calcium
436 isotope or a dye such as alizarin (Lamberts, 1974) would provide an estimate for gross
437 calcification. This dual isotopic approach could allow the simultaneous and direct assessment of
438 rates for GC and dissolution on the same coral. The importance of accurately measuring these

processes, while considering the different factors that can affect the physiological response during incubation, emphasises the need for additional comparative studies to test the compatibility and accuracy of calcification estimates based on the TA and ^{45}Ca techniques in the context of OA research.

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Figure 1(on next page)

Conceptual framework of calcification in isolated coral colonies.

The specific calcification rate that determines the transition of calcium carbonate from its source to its sink is affected by the opposing effects of the biologically determined gross calcification rate and the dissolution rate. Depending on their respective magnitudes, the coral experiences net calcification or net dissolution of skeletal material. Quantifying net calcification in corals is commonly achieved by monitoring the change in source (via forms of titration) or sink (e.g., buoyant weight) material. When assessed over short time periods, where gross dissolution is negligible/not detectable, sensitive methods that can detect incorporated labels provide an estimate for gross calcification. Note that changes in skeletal density and porosity under low pH might alter gross calcification without involving processes of dissolution.

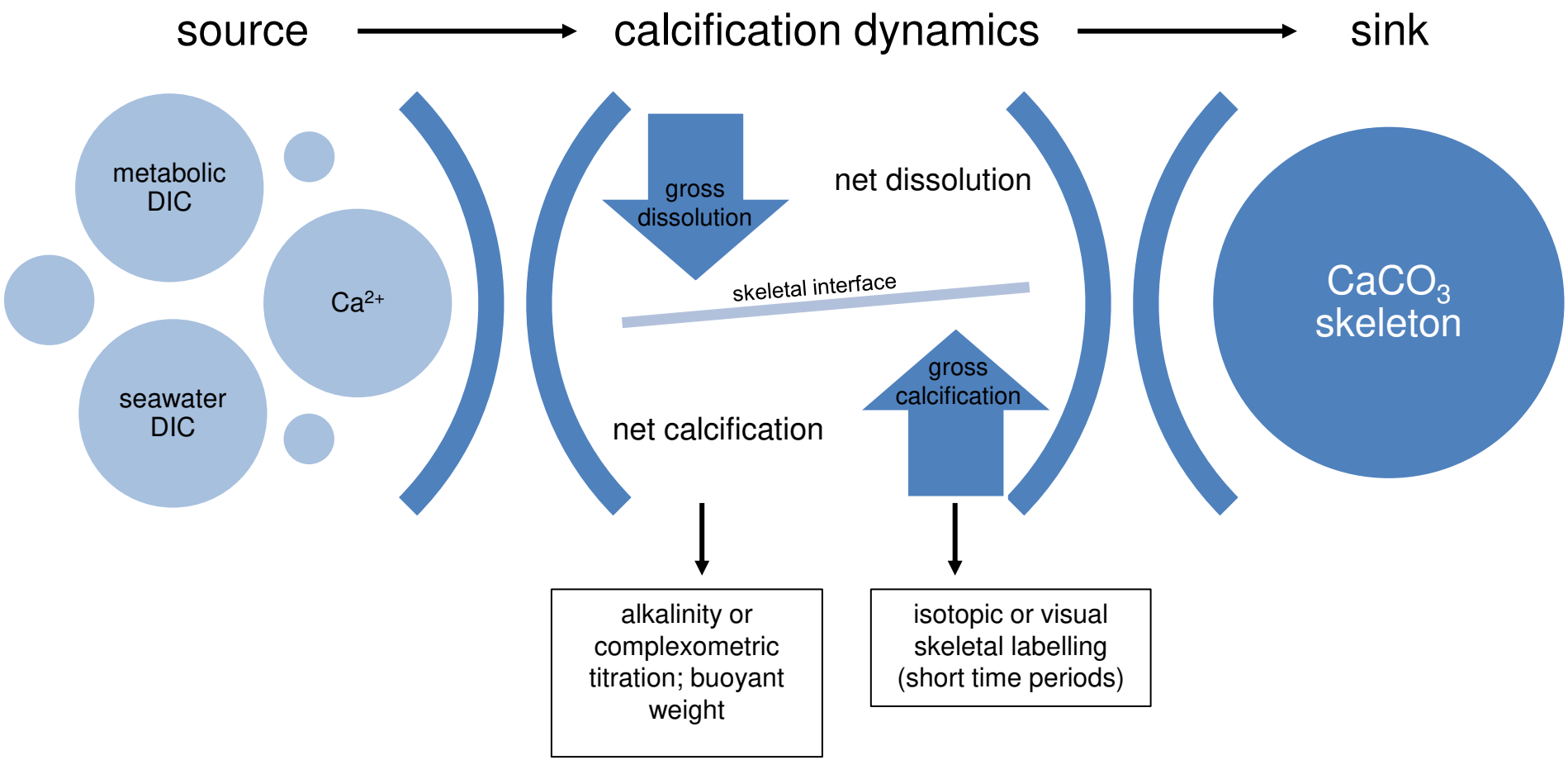

source
calcification dynamics
sink
metabolic DIC
Ca2+
seawater DIC
gross dissolution
net dissolution
skeletal interface
gross calcification
net calcification
CaCO3 skeleton
alkalinity or complexometric titration; buoyant weight
isotopic or visual skeletal labelling (short time periods)

Figure 2(on next page)

Calcification rates.

Calcification rates of *Stylophora pistillata* at pH_T 8.1 (A) and pH_T 7.5 (B) derived from different incubation intervals and measurement methods (^{45}Ca uptake *vs*. TA) as boxplots with median line (N = 6).

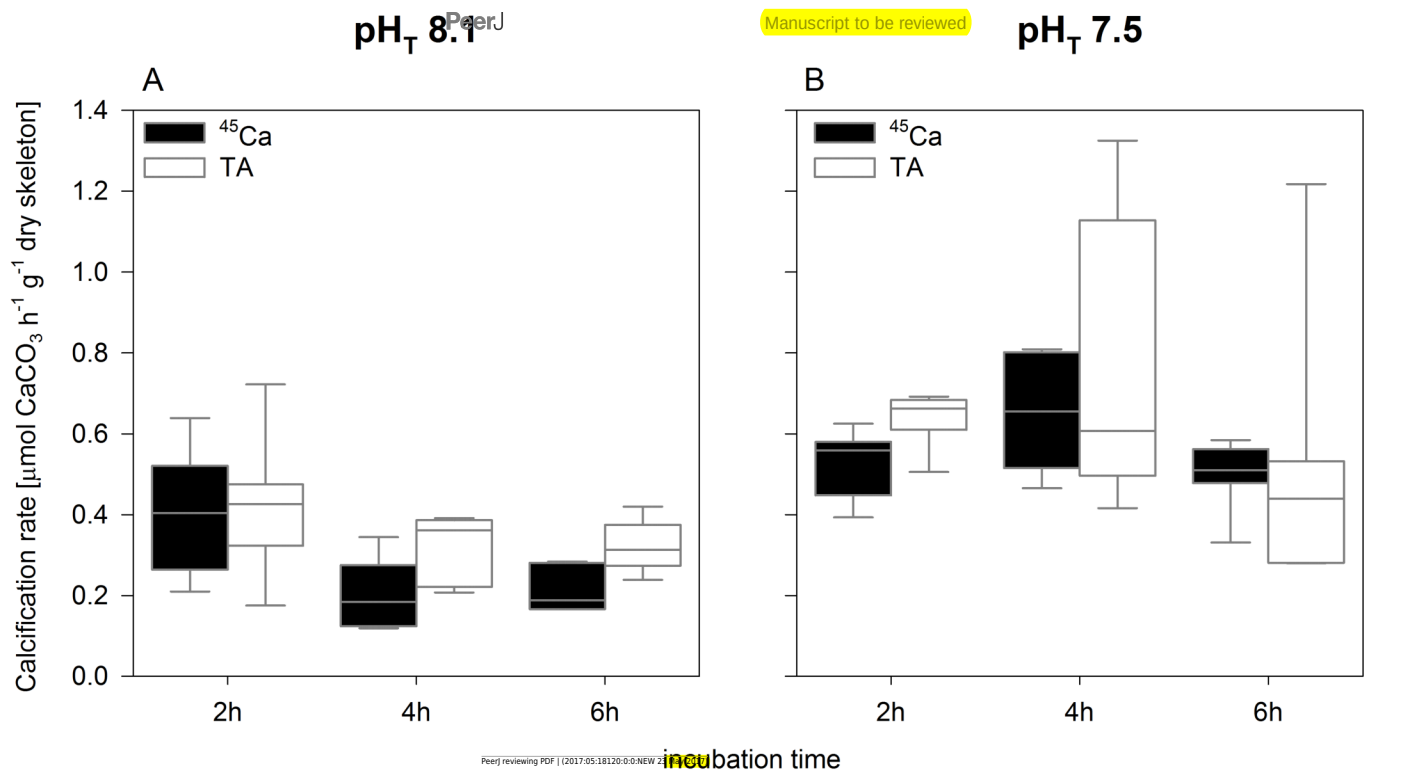
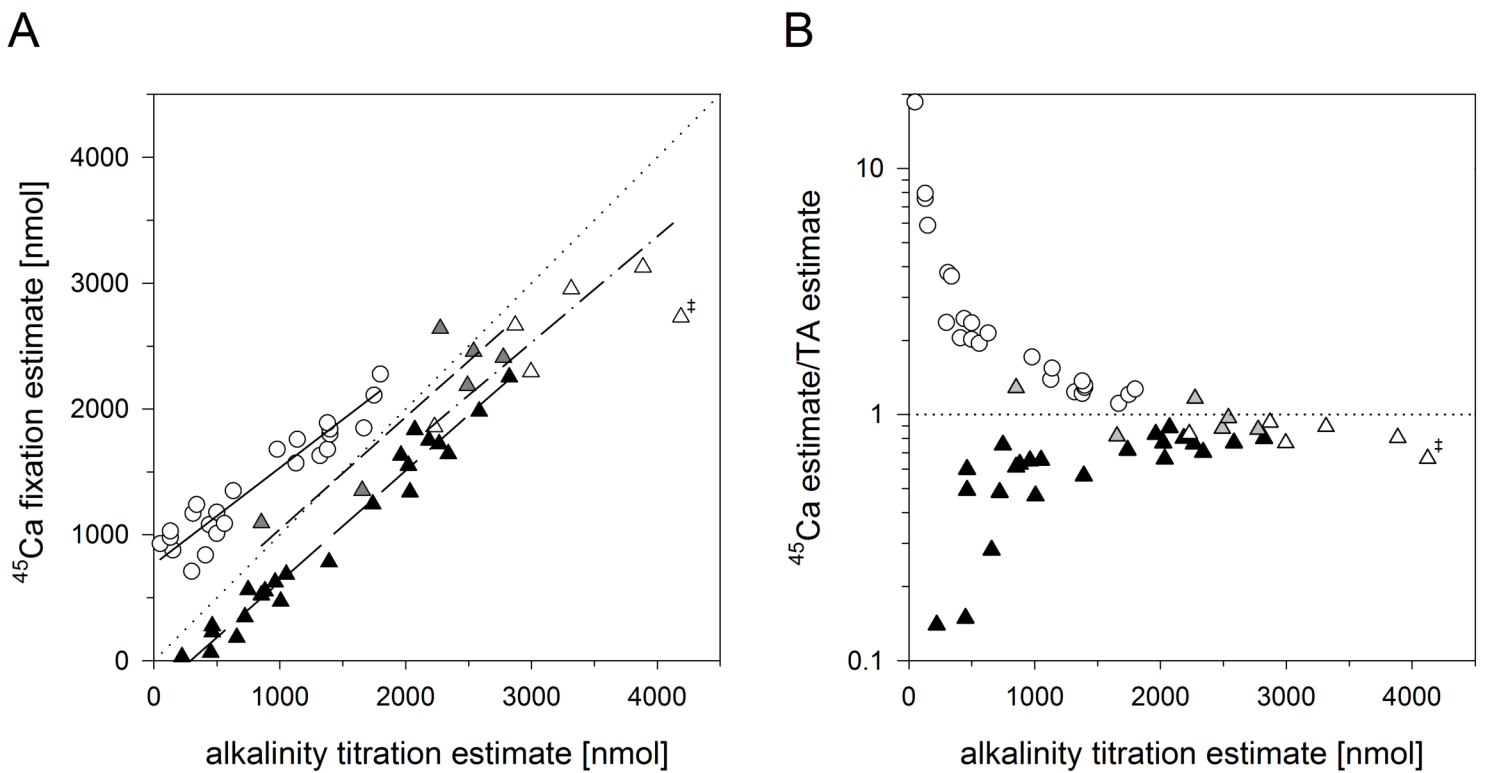

pH$_T$ 8.1
pH$_T$ 7.5
A
B
^{45}Ca
TA
Calcification rate [µmol CaCO$_3$ h^{-1} g^{-1} dry skeleton]
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
2h
4h
6h
incubation time

Figure 3(on next page)

TA vs ^{45}Ca estimates.

Relationship between calcification estimates based on TA titration and ^{45}Ca fixation for *Stylophora pistillata* (triangles; white and grey this study, black from Tambutté et al. 1995) and *Acropora formosa* (circle, Smith & Kinsey 1978). Geometric regressions are indicated for all datasets (Table S1) based on Smith & Kinsey (1978) (solid; after 30 min of incubation with *A. formosa*), Tambutté *et al*. (1995) (long dash 0.25-3h incubation with *S. pistillata*) and this study (short dash after 2h with *S. pistillata* pH_T 8.1, dash-dot pH_T 7.5). Statistical significance of pairwise correlations indicated by asterisks. Double dagger indicates excluded replicate point in the pH_T 7.5 dataset. Note that we assume that Smith & Kinsey (1978) and Tambutté et al. (1995) used blank corrected calcification estimates.



▲ *S. pistillata* ambient pH; y = 0.883x - 254.54; r = 0.99*
○ *A. formosa* ambient pH; y = 0.766x + 766.49; r = 0.95*
△ *S. pistillata* pH_T 7.5; y = 0.843x + 1.11; r = 0.92*
△ *S. pistillata* pH_T 8.1; y = 0.892x + 151.54; r = 0.90*

Table 1(on next page)

Methods for measuring calcification in tropical corals.

Overview of the most common methods and their employed acclimation and incubation times for measuring OA effects on calcification in tropical hermatypic corals. The detailed summary for each species and its approximate change in calcification is provided in Table S1. Note that the number of studies (in brackets) might not add up to the given total number, since some studies might have used different acclimation times when performing multiple experiments or involving different species. BW, buoyant weight; TA, total alkalinity.

Table 1. Overview of the most common methods and their employed acclimation and incubation times for measuring OA effects on calcification in tropical hermatypic corals. The detailed summary for each species and its approximate change in calcification is provided in Table S1. Note that the number of studies (in brackets) might not add up to the given total number since some studies might have used different acclimation times when performing multiple experiments or involving different species. BW, buoyant weight; TA, total alkalinity.

Method	No. of species	No. of studies	Acclimation time[#]	Incubation time
BW	32	26	< 1 month (10)	-
			1-2 month (13)	-
			3-12 month (5)	-
			> 1 year (2)	-
TA	17	13	no acclimation (5; 2[&])	< 2.5 hours (7)
			several hours (2)	3-7 hours (3)
			< 1 month (4)	> 12 hours (2)
			1-2 months (2)	
Ca isotopes	1	3	no acclimation (1)	3-7 hours (2; ^{45}Ca)
			1-2 months (2)	> 12 hours (1; ^{43}Ca*)
Other methods*	3	5	no acclimation (2; 1[&])	1-2 months (2)
			> 1 year (3)	> 1 year (1)

[#]Time required for acclimation and incubation time were summed up for coral calcification measured via BW

[&]Corals were already naturally exposed to low pH conditions

[*]e.g., lateral and cross-sectional growth, linear extension, skeletal density

Table 2 (on next page)

Seawater chemistry over 16 month acclimation period

Seawater carbonate chemistry in both pH treatments. TA and pH were measured, while the inorganic carbon speciation and aragonite saturation state (Ω_{arag}) were calculated based on pH and TA measurements, using the software CO2SYS version 1.03 (Lewis et al., 1998 ; Pierrot et al., 2006).

Table 2. Seawater carbonate chemistry in both pH treatments. TA and pH were measured, while the inorganic carbon speciation and aragonite saturation state (Ω_{arag}) were calculated based on pH and TA measurements, using the software CO2SYS version 1.03 (Lewis et al., 1998; Pierrot et al., 2006).

pH_T	TA [µeq kg^{-1}]	DIC [µmol kg^{-1}]	pCO_2 [µatm]	$CO_{2(aq)}$ [µmol kg^{-1}]	HCO_3^- [µmol kg^{-1}]	CO_3^{2-} [µmol kg^{-1}]	Ω_{arag}
8.09	2501	2122	387	10.6	1846	265	4.02
7.49	2499	2431	1917	52.0	2295	82	1.25

Table 3(on next page)

Changes in seawater carbonate chemistry over incubation time.

Values show absolute changes from the initial conditions (mean±SD, N=6). Values in brackets indicate relative changes to initial values to account for the shifted carbon equilibrium between both pH treatments.

Table 3. Changes in seawater carbonate chemistry over incubation time. Values show absolute changes from the initial conditions (mean±SD, N=6). Values in brackets indicate relative changes to initial values to account for the shifted carbon equilibrium between both pH treatments.

	pH 8.1			pH 7.5		
	2h	4h	6h	2h	4h	6h
Alkalinity (µeq kg^{-1})	-106±36	-152±48	-198±30	-164±37	-310±115	-345±98
	(-4%)	(-6%)	(-8%)	(-7%)	(-12%)	(-14%)
DIC (µmol kg^{-1})	-215±32	-458±59	-537±82	-277±76	-564±124	-791±103
	(-10%)	(-21%)	(-25%)	(-11%)	(-23%)	(-32%)
pH	+0.17±0.04	+0.41±0.06	+0.46±0.10	+0.29±0.11	+0.59±0.09	+0.89±0.06
	(+2%)	(+5%)	(+6%)	(+4%)	(+8%)	(+12%)
$CO_{2(aq)}$ (µmol kg^{-1})	-4.5±0.7	-8.0±0.6	-8.5±0.8	-34.6±10.9	-52.6±2.9	-60.0±1.2
	(-42%)	(-74%)	(-78%)	(-53%)	(-81%)	(-92%)
HCO_3^- (µmol kg^{-1})	-276±42	-636±80	-730±134	-293±87	-640±132	-974±109
	(-15%)	(-34%)	(-39%)	(-13%)	(-27%)	(-42%)
CO_3^{2-} (µmol kg^{-1})	+65±21	+185±33	+201±54	+51±22	+129±31	+244±30
	(+25%)	(+70%)	(+76%)	(+73%)	(+186%)	(+352%)

Table 4(on next page)

Statistical output.

Repeated measures ANOVA results for calcification rates determined from the TA or ^{45}Ca technique. Asterisks indicate significant results. Note that these rates are derived from fragments of a single biological replicate for the purpose of methodological comparison.

Table 4. Repeated measures ANOVA results for calcification rates determined from the TA or ^{45}Ca technique. Asterisks indicate significant results. Note that these rates are derived from fragments of a single biological replicate for the purpose of methodological comparison.

Complete dataset (2h, 4h, 6h)

	***F*-value**	**p-value**
Between subjects		
pH	$F_{1,30} = 4.565$	p = 0.0409*
incubation time	$F_{1,30} = 1.850$	p = 0.1748
pH x incubation time	$F_{1,30} = 2.581$	p = 0.0925
Within subjects		
method	$F_{1,30} = 3.117$	p = 0.0877
method x pH	$F_{1,30} = 1.210$	p = 0.2801
method x incubation time	$F_{1,30} = 0.690$	p = 0.5093
method x pH x incubation time	$F_{1,30} = 2.484$	p = 0.1004

2 h dataset

Between subjects		
pH	$F_{1,10} = 110.748$	p < 0.0001*
Within subjects		
method	$F_{1,10} = 20.020$	p = 0.0012*
method x pH	$F_{1,10} = 7.152$	p = 0.0233*