

Unexpected results from direct measurement, with a torsion microbalance in a closed system, of calcification rates of the coral *Agaricia agaricites* (Scleractinia:Agariciidae) and concomitant changes in seawater pH

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Received 15-VIII-2013 Corrected 23-II-2014 Accepted 24-III-2014

Abstract: Ocean acidification is impacting the calcification of corals, but the mechanisms of calcification are still unclear. To explore the relationship between calcification and pH, small pieces of coral were suspended from a torsion microbalance in gently stirred, temperature controlled, seawater in a closed chamber. Net calcification rate and pH were continuously monitored while light, temperature or pH could be manipulated. The coral pieces were from the edges of thin plates of *Agaricia agaricites* and were studied alive and freshly collected. Unexpectedly, when calcification was taking place ($n=9$, $0.082 \text{ mg}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$), as determined by weight increase, the pH of the surrounding seawater medium changed little ($n=10$, $-0.0047 \text{ pH units}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$). When calcification was not taking place the decrease of seawater pH was an order of magnitude higher, $-0.013 \text{ pH units}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$. This is the opposite of what is expected when calcium carbonate (CaCO_3) forms. Similarly, fresh skeleton initially showed no change of pH in the seawater medium although the rates of weight gain were high (upto $1.0 \text{ mg}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$). After 10 hours, as the rate of deposition decreased following a generalized Michaelis-Menten growth curve, the pH began to decrease dramatically indicating an increase of CO_2 in the seawater. These unexpected results can be explained if unstable calcium bicarbonate ($\text{Ca}(\text{HCO}_3)_2$) is formed in the organic matrix/carbonic anhydrase surface and slowly transforms later to CaCO_3 . Pieces of living coral monitored in the chamber for 30 hours gained weight during the day and lost it at night. The loss would be consistent with the transformation of $\text{Ca}(\text{HCO}_3)_2$ to CaCO_3 with the release of CO_2 . The mean calcification rate of live coral was greater ($n=8$, $p=0.0027$) in high light ($120 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) at $0.098 \text{ mg}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$, compared to $0.063 \text{ mg}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$ in low light ($12 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$). However, at the same time the mean rate of pH change was -0.0076 under low light compared to -0.0030 under high light ($n=8$, $p=0.0001$). The difference can be explained by CO_2 being used for photosynthesis by zooxanthellae. The deposition rate of live coral was not affected by the addition of phosphate but the rate of weight gain by the freshly collected skeleton was strongly enhanced by phosphate. These results indicate that care should be applied in the application of the alkalinity anomaly technique for the measurement of calcification in corals. Rev. Biol. Trop. 62 (Suppl. 3): 25-38. Epub 2014 September 01.

Key words: coral calcification, CO_2 , pH, organic matrix, carbonic anhydrase, $\text{Ca}(\text{HCO}_3)_2$.

The processes involved in the production of an aragonite (CaCO_3) skeleton in corals are poorly known compared compared to calcification in other animals (Allemand, Tambutté, Zoccola & Tambutté, 2011). Seawater contains about $10.3 \text{ mmol}\cdot\text{kg}^{-1}$ of calcium ions (Ca^{2+}), dissolved inorganic carbon is present as carbon dioxide (CO_2), carbonic acid (H_2CO_3), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), with the equilibria between them described by their

equilibrium constants and their relationships with pH, alkalinity and temperature. It seems to have been taken for granted that the formation of aragonite crystals would result from the combination of Ca^{2+} and CO_3^{2-} . However, Lee, Park, Kwak & Cho (2010) have reported crystalline aragonite deposition from a solution of CaCl_2 containing carbonic anhydrase using CO_2 directly from the atmosphere. The sources of calcium and carbon and how they



reach the calcification site have been reviewed by Cohen and McConnaughey (2003), Furla, Galgani, Durand and Allemand (2000) and Allemand et al. (2004; 2011). Carbon dioxide which can move freely through cell membranes was proposed by McConnaughey (1989) as a substrate for calcification to account for ^{18}O and ^{13}C deficiencies in coral skeletons (McConnaughey, 2000). The response of corals to changes of CO_2 partial pressures and temperature was reviewed by Reynaud et al. (2003). While calcification and photosynthesis may compete for the same DIC pool they are sometimes regarded as complementary (Gattuso, Allemand & Frankignoulle, 1999) rather than in competition (Langdon & Atkinson, 2005). With a focus on ocean acidification as the result of increased anthropogenic carbon dioxide in the atmosphere, calcification has been increasingly linked to the calcium-carbonate saturation state (ω) which is the ratio of the ion concentration product ($[\text{Ca}^{2+}] \times [\text{CO}_3^{2-}]$) to the solubility product of the mineral deposited, in this case aragonite (Allemande *et al.* 2004). As acidity increases the relative concentration of carbonate in seawater is reduced and has been used to predict decreased calcification at the organism and community levels (Kleypas et al., 1999; Gattuso et al., 1999; Marubini & Thake, 1999; Langdon, 2000; Langdon et al., 2000; Langdon, Broecker & Hammond, 2003; Erez et al., 2011).

The mechanisms of calcification have been fully reviewed by Allemand et al. (2011). Active calcification by the coral involves secretion of a layer of organic matrix (reviewed by Allemand, Tambutté, Girard & Jaubert, 1998 and Allemand et al., 2011). Sandeman (2012) presented evidence that carbon dioxide is the probable substrate for calcification and that carbonic anhydrase present in organic matrix is the basis for calcification that takes place on the surface exposed after removal of living tissue by waterpiking. Deposition of calcium carbonate crystallized in the form of aragonite takes place from the extracellular calcifying fluid (ECF) or hydrogel-like medium (ECM) (Bryan & Hill, 1941) on an organic matrix

framework on the surface of the skeleton underlying the calicoblastic layers. What is still not clear is in what form dissolved inorganic carbon (DIC) reaches the site of calcification, and to what extent, if at all, the calcifying surface is in direct contact with seawater. Charged ions such as Ca^{2+} , HCO_3^- and CO_3^{2-} cannot move passively through cell membranes so either active transport via a transcellular route or passive diffusion of ions or seawater using a paracellular route at the boundaries of cells or some combination of these routes may be involved (Allemand et al., 2011). The median value of enhanced calcification in light (LEC) is around 3 (Gattuso et al., 1999). The range of ratios observed is large and Allemand et al. (2011) reviewed the possible mechanisms by which calcification is enhanced. The answers to these questions are complicated by the fact that zooxanthellae in the tissues take up CO_2 for photosynthesis and CO_2 is released as the result of respiration by coral tissue and their symbionts. Sandeman (2012) presented evidence that CO_2 is the substrate for calcification by living and freshly waterpiked coral skeleton and that the enzyme carbonic anhydrase appears to be incorporated in the organic matrix secreted by the calicoblastic layers. It was also shown that for dead coral skeleton in sea water CO_3^{2-} is the substrate and at $\text{pH} > 7.4$ the skeleton gained weight and at $\text{pH} < 7.4$ dissolved.

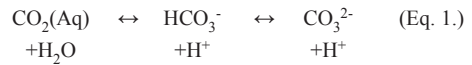
Calcification rates of corals are commonly derived by buoyant weighing techniques (Davies, 1989), uptake of the radioactive isotope ^{45}Ca (e.g. Moya et al., 2006; Al-Horani, Tambutté & Allemand, 2007), the alkalinity anomaly technique (Smith & Key, 1975) or using a sclero-chronological technique (e.g. by Gischler & Oschmann, 2005). Each of these methods has its advantages and disadvantages, with some requiring destruction of the coral. The buoyant weighing technique used by Franzisket (1964) and developed by Davies (1989) for nubbins of *Porites porites* has been used to measure calcification rates over time periods of less than a day but a disadvantage in physiological studies is that the coral or a coral 'nubbins' has to be transferred to and

from a balance for weighing. For physiological experiments over shorter time periods it is desirable to minimise any physical disturbance and provide stable conditions while still being able to change experimental parameters such as light, temperature or pH. Sensitive balances are expensive, sensitive to sea air and because they tend to drift they may require re-zeroing regularly which requires disturbing the organism. Davies (1989) established with his buoyant weighing technique that for corals changes in the tissue weight over the time span of an experiment were small and could be corrected for, and for imperforate corals such as thin plates of *Agaricia* could be ignored.

In an earlier paper Sandeman (2012) reported on a torsion balance developed to investigate calcification in small pieces of the coral *Agaricia agaricites*. A major disadvantage of the technique was that in an open system, the seawater medium could exchange CO₂ with the air, changing the pH and bringing uncertainty to the carbon chemistry. In this study the torsion balance has been completely enclosed in a sealed chamber to avoid the exchange of CO₂ with the air. The pH electrodes and their associated temperature sensors have been incorporated so that pH can be continuously monitored while calcification takes place. In order to seal off the system from the air some modifications were made to the balance. The torsion wire was of tungsten and shortened considerably. The beam also had to be shorter in order to fit the container and, to regain some of the lost sensitivity, thinner (0.025mm) wire was used. The lighter the beam of a balance the higher is the sensitivity (Vogel, 1961) and it was found that a hollow, sealed, carbon fibre beam, sealed at its ends, provided buoyancy making the beam assembly lighter under water and greatly increasing the sensitivity of the balance.

Seawater contains about 10.3mmol.kg⁻¹ of calcium ions (Ca²⁺), dissolved inorganic carbon is present in seawater as carbon dioxide (CO₂), carbonic acid (H₂CO₃), bicarbonate (HCO₃⁻) and carbonate (CO₃²⁻), the equilibria

between the species of carbon can be represented as follows:



The equilibria between the species of carbon are governed by their equilibrium constants and their relationships with pH, alkalinity and temperature. These relationships can be seen in Fig. 1 which is similar to Fig. 1. 1. 3 in Zeebe and Wolf-Gladrow (2000) but is for seawater (S= 35.5 and T= 28°C) used in this study. In general, at normal seawater pH (8-8.2), the bulk of the carbon is present as HCO₃⁻ with a small quantity as CO₃²⁻ and even smaller quantity as CO₂. If, as in the acidification of sea as the result of increased atmospheric carbon dioxide, there is an increase in dissolved CO₂, the total dissolved organic carbon (DIC) increases but total alkalinity (TA) does not change and a decrease in pH results (Fig. 1). If CO₂ in the system decreases the opposite happens, DIC decreases and TA remains the same and there is a consequent increase in pH. If

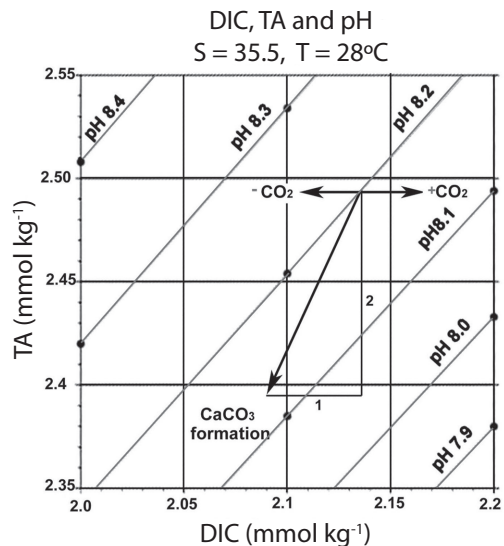
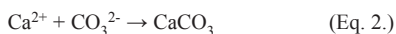


Fig. 1. Relationship between DIC, TA and pH in seawater (S=35.5, T= 28°C) showing the effect of CaCO₃ formation and of taking up or losing CO₂. Calculated with CO@SYS program.

calcification takes place with the formation of calcium carbonate as a result of the following reaction (Zeebe & Wolf-Gladrow, 2000):



DIC decreases by 1 unit and TA decreases by two units, (as calcium ions have a double positive charge). The new equilibrium position, as seen in Fig. 1, results in a lower pH. If CaCO_3 dissolves the direction of the arrow is reversed and there is an increase of 1 unit in DIC and two units of TA and the pH increases. The change in equilibrium point for CaCO_3 formation is the same whether the source of carbon is CO_3^{2-} , HCO_3^- or CO_2 (Zeebe & Wolf-Gladrow, 2000).

The aim in this study was use the improved torsion balance to follow up on the rather extraordinary findings related to freshly water-picked *Agaricia* skeletons (Sandeman, 2012) and to further explore the indications that calcification in the living coral might involve CO_2 rather than CO_3^{2-} . Generally two of DIC, TA, pH and $[\text{CO}_2]$ are required to calculate the components of the carbonate system in seawater. The experimental set-up used here permits continuous monitoring of pH change and weight change of the coral. If the weight change is mainly of skeleton formation or dissolution then this permits estimates of changes of both DIC and TA. The closed chamber, however, does not permit TA to be measured other than at the beginning and end of an experiment and was not attempted. It was also hoped that under different lighting conditions insight might be gained on the mechanism involved in the enhancement of calcification of corals by light.

MATERIALS AND METHODS

Small pieces of *Agaricia agaricites* were snipped from the edge of thin plates of young colonies growing near the reef crest opposite the Discovery Bay Marine Laboratory. The pieces were immediately transported to the seawater tables where they were trimmed to a

suitable size (1-2cm²) then suspended, in a horizontal orientation, by loops of thin (0.025mm diam.) polyester monofilament in gently flowing seawater in a seawater table. The pieces were used in experiments within an hour of collection. There is some indication that orthophosphate in low concentrations inhibits the deposition and dissolution of calcium carbonate (Pytkowicz, 1973; Morse, 1974; Burton & Walter, 1990) and for live corals and freshly water-picked coral skeleton the seawater used in the experimental chambers was collected from at least a mile offshore. This proved to have the lowest phosphate levels as measured by the method for reactive phosphorus in Strickland and Parsons (1965). For experiments with dead corals "Instant Ocean" artificial seawater which has low (0.05µmol) phosphate concentration (Atkinson & Bingham, 1999) was used. All seawater was millepore filtered (0.45µ) immediately before use. Salinity was measured with a Pinpoint Salinity Monitor (American Marine Inc.). Total Alkalinity of the seawater was estimated using the method of Smith and Kinsey (1978). The relationships between DIC, TA and pH and $[\text{CO}_2]$, $[\text{CO}_3^{2-}]$ and ω for each batch of seawater (Table 1) were estimated using the CO@SYS program. From the initial salinity, temperature, total alkalinity (TA) and pH for each batch of seawater the total dissolved carbon (DIC) was obtained. The experimentally measured change of weight of the skeleton then permits calculation of the carbonate withdrawn from the seawater medium and estimation of DIC at the end of each experiment.

The balance (Fig. 2) chambers (volume ≈ 1L) were filled with millepore filtered seawater brought to the temperature at which the experiment was to be conducted (27°C). Temperature sensing thermistors, control circuits, and insulated heater coils maintained a constant temperature within 0.2 °C. Small magnetic stirrers (1.0x0. cm) gently and continuously circulated the water in the chambers. The pH could be changed by exchanging some of the seawater in the chamber with seawater that had a high dissolved CO_2 content using a syringe through the hole in the centre of the

TABLE 1
Estimated carbon parameters for seawater used

Parameter (Salinity=35.5, Temp. 28°C)	Natural seawater (1km offshore, Discovery Bay)	Artificial seawater (Instant ocean)
Total Alkalinity (TA), $\mu\text{eq kg}^{-1}$	2 490	2 560
pH (NBS)	8.18	8.21
Total Carbon (DIC), $\mu\text{mol kg}^{-1}$	2 029	1 911
[CO ₂], $\mu\text{mol kg}^{-1}$	10.8	9.5
[HCO ₃ ⁻], $\mu\text{mol kg}^{-1}$	1 781	1 665
[CO ₃ ²⁻], $\mu\text{mol kg}^{-1}$	236	237
Ω	5.7	5.7

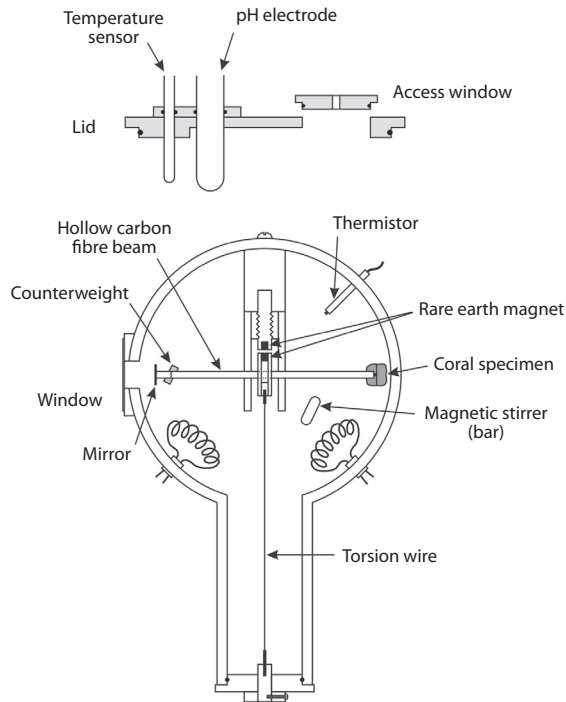


Fig. 2. The torsion balance. The ends of a 15cm length of torsion wire (tungsten, diam. 0.05-0.11mm) are embedded in short pieces of stainless steel tubing (0.5mm OD) with cyanoacrylate. One end is held by friction fit in a polyethylene cylinder which can be rotated in its mount (torsion wire adjustment). The other end of the torsion wire is inserted into a neoprene cylinder at one end of a 1.5cm tube (diam. 0.63cm) of plexiglass with a cylindrical rare earth magnet (0.31 x 0.31cm diam.) embedded in its other end. The wire is held under tension by a second similar magnet embedded in the end of a threaded 6.3mm diam. plexiglass rod. This rod can be turned to change the separation between the two magnets. Both magnets are covered by thin plastic so that they are not in contact with the seawater. The hollow carbon fibre tubular beam has both ends sealed and is inserted through a hole drilled through the plexiglass cylinder at right angles to the torsion wire. One end of the beam has a small tungsten wire hook to support the specimen the other end has a small (4x8mm) piece of thin cover glass cemented on the end, at right angles, which acts as a mirror. The other end of the beam supports a small weight that can be slid along the beam and acts as a counterbalance. The wall of the chamber has a thin glass window near the mirror. The beam from a laser pointer is directed through the window and is reflected by the mirror back through the window onto a scale at a distance of about 3 m. This enables small angles of rotation of the torsion wire to be detected and measured. The lid of the chamber has an O-ring seal, holes with O-ring seals for a pH electrode and temperature sensor and an access window above the specimen and closure also with o-ring seal. The centre of the closure has a hole through which seawater can be or air bubbles released.

access window. For higher pH levels water in the vessel was exchanged with seawater that had been bubbled with CO₂ free air. Two pH meters (Omega, PHB-212) with PHE-210 glass or PHE-1411 double junction electrodes, calibrated daily before experiments, using Omega PHA-4, PHA-7, and PHA-10 buffers, were used to monitor changes of pH in each balance chamber. Although the NBS scale is not recommended for use with sea water (Zeebe & Wolf-Glarow, 2000) this study is only concerned with changes in pH rather than absolute pH levels. Room lighting was 12-15 μmol.s⁻¹.m⁻² and additional light was provided by a metal halide spotlight (Philips 25W, 10°) above the balance chamber. Irradiance levels were measured with a LI-COR Quantum/Radiometer (LI-250).

Pieces of coral were carefully inspected before each experiment and were rejected if there were signs of damage to the living tissue on the upper or lower surfaces or if mucus was present. A piece of coral was suspended by its monofilament loop from a hook at the end of the carbon fibre beam of the torsion balance (Fig. 2). The beam was balanced by sliding the counterweights along the other arm of the beam so that the reflected spot from the laser pointer was in the appropriate part of the scale. Further adjustment of the laser spot could be made with the torsion wire adjustment (Fig. 2). The lid with the pH and temperature probe was placed on the chamber and pressed into place so that its o-ring sealed the system, air bubbles were removed and sea water added through the access window. Finally the access window closure was put in place and its centre hole covered with a glass cover-slip and silicone grease seal. Change in weight as aragonite skeleton is deposited result in movement of the laser beam spot on the scale and readings were taken at intervals of about ten minutes. The course of an experiment was followed by plotting the readings on graph paper. Rates of weight gain or loss were calculated by regression analysis of series of 4-6 readings. Readings of pH were also taken at the same time. After a set of control reading a single experimental

parameter (irradiance, temperature or pH) could be changed and after an hour for acclimation a new set of readings could be taken to give a new rate. When rates of change were very low, longer periods between reading and more readings were taken. When calcification rates were high, as in the experiments involving freshly waterpiked coral, thicker (0.11mm) tungsten wire was used in the balance.

A series of small solid aragonite cubes (1-3mg) were prepared beforehand, these were cut from *Agaricia* skeletons, and their dry weights were accurately known. Each cube was attached to a 20 cm length of extremely fine monofilament consisting of a single strand from dental floss. Attachment was by dipping the end of the filament into cyanoacrylate and touching it to the cube. An aragonite cube was used to calibrate the system at the end of each experiment by dropping it, suspended by its monofilament, through the access window, carefully onto the coral's surface. The position of the laser spot on the scale with and without the aragonite cube in position was recorded. This was repeated several times and the mean displacement for the cube was calculated. From this the equivalent dry wt. of aragonite per scale unit could be calculated. The aragonite cubes were also used to verify the linearity of the scale. A commercial dental waterpik that had been modified with a narrower jet and to work at higher pressure was used to blast away the living tissue with a jet of seawater (water-piking). This exposes organic matrix and most recently deposited aragonite on the surface of the skeleton. Inspection with a dissecting microscope established that tissue was completely removed even from the deepest polyp cavities. Waterpiked skeletons that had been soaked in seawater for at least 72 hours were treated in experiments as "dead coral". Coral surface areas were estimated with aluminium foil (Marsh, 1970). NCSS statistical software (Number Crunching Statistical Systems, Dr Jerry Heintze, Kaysville, Utah) was used for obtaining a best fit for the growth curves.

RESULTS

Waterpiked coral: Freshly waterpiked corals showed the pattern of weight gain reported previously (Sandeman, 2012). In a typical example (Fig. 3A) the initial rate (first hour) of weight gain of $0.97 \text{ mg}\cdot\text{h}^{-1}$ decreased following the generalized Michaelis Menten growth curve (Lopez et al., 2000; Sandeman, 2012) for the first 15 hours then maintained a steady rate of increase of $0.072 \text{ mg}\cdot\text{hr}^{-2}\cdot\text{cm}^{-2}$ until the termination of the experiment ten hours later. During the initial phase the pH of the seawater remained constant while the deposition rate changed from an initial $0.99 \text{ mg}\cdot\text{hr}^{-1}$ to a constant rate of $0.072 \text{ mg}\cdot\text{hr}^{-1}$. However, as the changeover point was reached the seawater pH started to drop and eventually reached a steady rate of change of $0.0047 \text{ pH units}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$. The mean initial calcification rate ($n=4$), over the first hour, was $0.84 \text{ mg}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$ with the pH changing at $-0.00041 \text{ units}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$. After reaching a steady rate of increase the mean calcification rate was $0.036 \text{ mg}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$ and the pH rate of change increased by an order of magnitude to $0.0030 \text{ pH units}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$.

Over the pH range of 6.9-8.6 aragonite growth rates have been shown to decrease with increasing PO_4^{3-} (Burton & Walton, 1990). Experiments to see how freshly waterpiked coral responded to $50 \mu\text{mol}$ of orthophosphate gave the surprising result that rather than being inhibited the deposition rate on waterpiked coral (Fig. 3B) increased by over an order of magnitude from $0.04 \text{ mg}\cdot\text{h}^{-1}$ to $0.88 \text{ mg}\cdot\text{h}^{-1}$. The experiment was repeated four times with similar results.

Live coral: When placed in the sealed chamber live corals commonly showed no weight increase for the first few hours then started calcifying at a steady rate. During the period when no calcification was taking place the pH of the chamber decreased steadily. After calcification started, as indicated by weight increase gain and during calcification the pH in the chamber remained nearly constant. For example, for coral #8.2 (Fig. 4A) during the

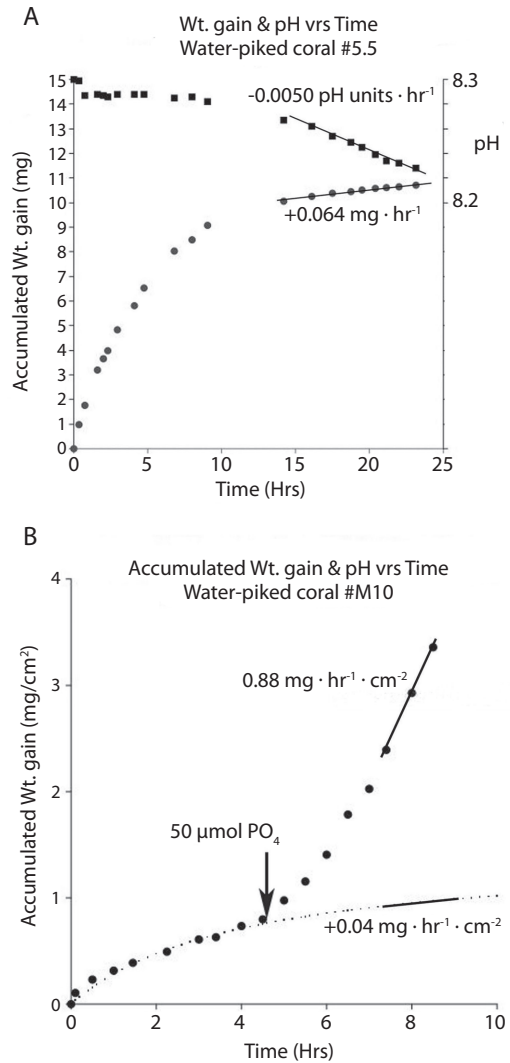


Fig. 3. Freshly water piked *Agaricia* skeleton, Slopes calculated by regression analysis. A: Accumulated weight and pH versus time. B: Accumulated weight gain versus time. Predicted weight gain from the best fit curve (Sandeman, 2012) for $W_t = (W_{\text{max}}\cdot t)/(K+t)$ compared to actual weight gain after spiking with orthophosphate.

initial period with no calcification taking place the pH decreased by $0.0116 \text{ pH units}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$. After calcification started ($0.058 \text{ mg}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$) the pH remained nearly constant, decreasing at the much lower rate of $0.0011 \text{ pH units}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$. In seawater with a mean pH of 8.173 when no calcification was taking place the mean pH change ($n=10$) was -0.013 pH units .

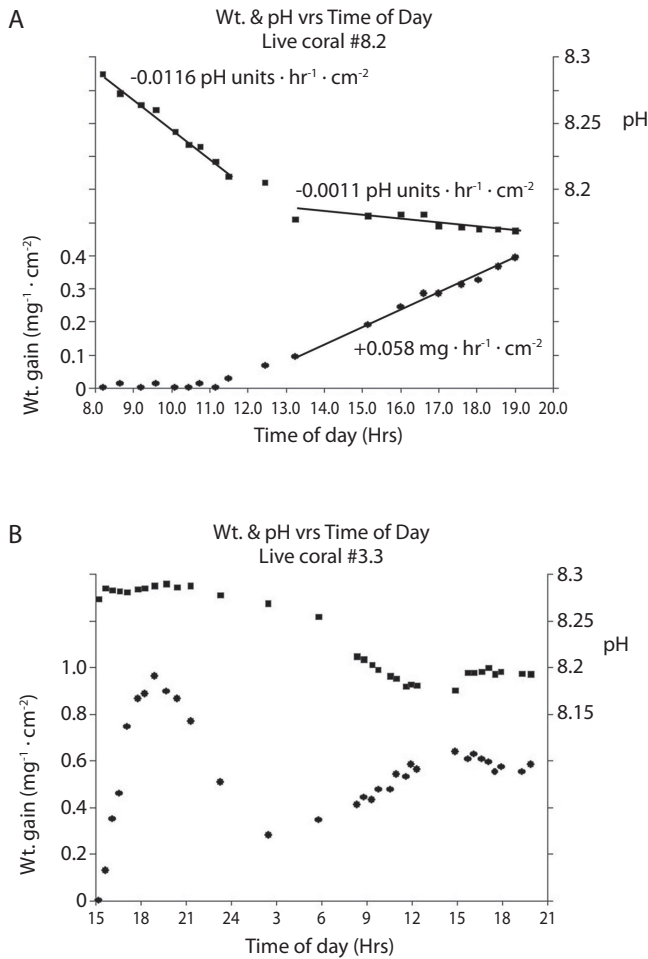


Fig. 4. Live *Agaricia*, A: Showing weight change and pH versus time. Rates calculated by regression analysis. B: Weight and pH change plotted against time of day over a period of 30 hrs.

$\text{hr}^{-1} \cdot \text{cm}^{-2}$. After calcification started the mean calcification rate of the corals was $0.082 \text{ mg} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$ and the pH rate of change decreased to a mean of $-0.0047 \text{ pH units} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$.

A piece of *Agaricia* placed in the balance chamber at 3pm and kept for 30 hours (Fig. 4B) showed calcification taking place ($0.348 \text{ mg} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$) until 7 pm coupled with small changes in pH. Thereafter the coral lost weight ($-0.94 \text{ mg} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$) and the pH decreased ($-0.0019 \text{ pH units} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$) until about 3 am. Weight gain commenced again and continued until about 3pm, but this time the pH decreased at a higher rate. The weight after 24

hours did not reach its previous level. Similar results were obtained when the experiment was repeated.

Mean calcification rates obtained under low laboratory lighting ($12 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) were $0.063 \text{ mg} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$ as compared to $0.098 \text{ mg} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$ under higher light ($n=8$, $p=0.0027$). At the same time the pH change under low light was $-0.0076 \text{ pH units} \cdot \text{hr}^{-1}$ while under higher light was lower at $-0.0030 \text{ pH units} \cdot \text{hr}^{-1}$ ($n=8$, $p=0.0001$).

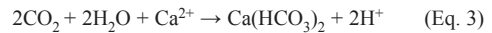
Dead coral: The relationship between calcification rate and pH was explored and the

results presented in an earlier paper (Sandeman, 2012). When the experiments were repeated with pH monitoring the results obtained were exactly as expected from Zeebe and Wolf Gladrow (2000) and Fig. 1. When the rate of weight change was positive, i.e. when deposition of calcium carbonate was taking place, the concomitant pH change in the seawater medium was always, as expected (Fig. 1), in a negative direction and when dissolution of the coral skeleton took place the pH of the seawater medium always increased.

DISCUSSION

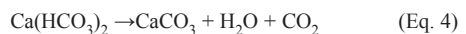
Waterpiked coral: Freshly waterpiked coral provides a simpler system than live coral in that calcification takes place directly on the surface of the organic matrix/skeleton which contains the enzyme carbonic anhydrase and which is in direct contact with the sea water medium (Sandeman, 2012) and is not separated by layers of tissue. The very high initial rate of calcification (mean, $0.84\text{mg}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$) was always accompanied by relatively small changes in pH of the seawater medium (mean $0.0004\text{ pH units}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$). During the first 10 hours the calcification rate dropped by an order of magnitude but the pH hardly changed. If CaCO_3 was formed, the pH of the seawater would have decreased rather than remain constant as the CaCO_3 arrow follows the reduction of DIC by one unit and TA by two units. Second, the rate of pH change would be proportional to the calcification, starting with a high rate and decreasing as the calcification rate decreases. The results obtained here cannot be reconciled with the conditions described in Zeebe and Wolf-Gladrow (2001) which are clearly set out “Precipitation of 1mol CaCO_3 reduces DIC by 1 mol and TA by 2mol independent of the carbon source (HCO_3^- , CO_3^{2-} , or even CO_2) used by the organisms for calcification”. A possible resolution to the problem is that the “ CaCO_3 formation” arrow (cf. Fig. 1) would follow the line for pH 8.2 if the DIC:TA ratio was 2:2. A possibility is that in the initial phase of exposure of the freshly waterpiked

skeleton the following reaction takes place on the surface with carbonic anhydrase resulting in the formation of the unstable calcium bicarbonate:



The reaction of two moles of carbon with one mole of Ca^{2+} would fulfill the requirement of a 2:2 ratio for DIC: TA to remain on the iso pH line (cf. Fig. 1).

After the initially very high rate of weight gain of the freshly waterpiked coral the rate decreases steadily but the pH remains the same indicating the same process is taking place during that period. In the second phase the pH begins to decrease and the weight gain reaches a steady rate. During this second phase the mean deposition rate was $0.036\text{ mg}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$ and the mean rate of pH change was $0.003\text{ pH units}\cdot\text{hr}^{-1}\cdot\text{cm}^{-2}$ which is an order of magnitude greater than the expected rate of pH change. However if, during this second phase, the calcium bicarbonate was slowly converting to calcium carbonate following the reaction:



Then the resulting release of CO_2 into the seawater would help to explain its high rate of pH decrease. The conversion of $\text{Ca}(\text{HCO}_3)_2$ to CaCO_3 would mean a decrease in weight of the skeleton but this was not evident in the results and it seems likely that as earlier experiments showed (Sandeman, 2012) deposition of CaCO_3 is taking place from seawater at the same time following Eq. 2. Calcium bicarbonate is only known in solution and has not previously been suggested as a precursor to carbonate formation in corals. It appears quite commonly in the geological literature as a precursor to calcium carbonate deposition in nature and its formation when carbonate solutions are bubbled with CO_2 is widely used for the experimental deposition of calcium carbonate. For example it was used in crystallization studies (Reddy, Plummer & Busenberg, 1981) and in a study of the kinetics of aragonite formation

(Romanek, Morse & Grossman, 2011). It has also been used in investigations of the conditions under which vaterite, calcite or aragonite are deposited and the effect of metallic ions on deposition (Tokuyama, Kitano & Kanamori, 1973; Kitano, Tokuyama & Arakaki, 1979). Of some interest is their finding that the presence of Mg^{2+} ions inhibits calcite formation and promotes aragonite formation. Calcium bicarbonate has also been used in the production of coccolith-like hollow shells by Walsh and Mann (1995) and in the growth of crystals on chitosan by Zhang et al. (2006). While the suggested formation of calcium bicarbonate might explain some of the experimental results obtained in this study further work will clearly have to be carried to confirm any role it might have in coral calcification.

The finding that phosphate enhanced rather than inhibited the deposition rate on the freshly waterpiked aragonite skeleton was very surprising. It seems unlikely that the weight increase could be the result of deposition of the added phosphate because in some experiments the weight deposited (up to 10mg) on the coral exceeded the weight of the added phosphate (50 μ mol) and the deposition rates as shown by the slope of the curves showed no signs of depletion of phosphate in the medium. Phosphate has been found to enhance the catalytic activity of human carbonic anhydrase III (Paranawithana, Tu, Laipis & Silverman, 1991) so the rather unexpected finding that phosphate enhances calcification in a freshly waterpiked coral adds weight to the conclusion (Sandeman, 2012) that carbonic anhydrase is indeed incorporated in the organic matrix of *Agaricia*. There is also a possibility that, if present, phosphate could have a role in calcification by enhancing the activity of carbonic anhydrase and amplifying the formation of skeleton where the quantities of carbonic anhydrase are highest. The results seen in Fig. 4 also suggest the shape of the Michaelis-Menten curve reaching an asymptote may not be the result of a limitation set by the concentrations of the reactants.

Live coral. In early stages of experiments with living coral (Fig. 4A) the seawater pH decreased steadily (-0.0116 pH units.hr⁻¹.cm⁻²) while the coral was not calcifying. When calcification was active the mean pH rate of change decreased by an order of magnitude to 0.0011 pH units.hr⁻¹.cm⁻². If carbonate is the substrate for calcification and the ratio of DIC:TA 1: 2 then the pH of the seawater should decrease during calcification and not change when the coral is not calcifying as indicated by weight changes taking place.

While calcification is taking place the pH of the medium remains nearly constant. This would suggest that DIC is being taken up and the TA is changing with a ratio of 2:2 and the same reaction could be taking place as was suggested for the freshly waterpiked skeleton surface. During periods when no calcification is taking place, there is no change in the calcium content of the sea water so the TA does not change. Following (Fig. 1) the decrease in pH must be the result of release by the coral of CO₂ into the seawater medium. Thus a possible explanation for the results obtained here is that calcium bicarbonate is deposited (Eq. 3) and that later the calcium bicarbonate is slowly converted to calcium carbonate (Eq. 4) accompanied by the release of CO₂.

The results from experiments in which corals were kept in the balance chamber, without the water being changed for 30 hours showed weight gain in the daylight hours followed by weight loss during the night. That the weight lost during the night was not recovered during the next day suggests that the corals were under stress and perhaps much weight should not be placed on this result. Decalcification in scleratinian corals is not common (Gattuso et al., 1999) but Kawaguti and Sakumoto (1948) reported output of Ca²⁺ in all corals exposed to dark. Tentori & Allemand (2006) reported decalcification and a daily cycle of sclerite formation in the soft coral *Cladiella*. While weight decrease took place (Fig. 4B) the pH of the medium decreased indicating a transfer of CO₂

from the coral into the seawater. This could be the CO_2 produced as the result of conversion of $\text{Ca}(\text{HCO}_3)_2$ to CaCO_3 , in which case a loss of 38% in weight might be expected.

How the materials necessary for calcification to take place reach the ECF is suggested in Fig. 5. CO_2 is uncharged and can diffuse through the plasma membranes of the coral tissues and be assisted in moving into the ECF by the CO_2 gradient which results from the activity of the Ca^{2+} ATPase pump. Protons generated (Eq. 3) on the skeleton surface in the ECF (Fig. 5) probably enter the calcicoblastic layer via the Ca^{2+} ATPase pump which moves calcium out of the tissues while moving protons in the other direction. Cohen and McConnaughey (2003) suggested that the calcium pump is light sensitive and there is some experimental evidence to support this (Sandeman, 2008a). There are several possibilities for what happen to protons thereafter. Although charged and existing in the form of hydronium ions (H_3O^+) in solution, in their hydrogen bonding, they have a unique conduction mechanism enabling them to hop from one water molecule to the next. They are able to move through membranes through water wires

or specific protein channels of which voltage gated channels, regulated by pH and voltage are the most efficient (DeCoursey, 2003). The voltage gated channels are directional, transfer protons out of cells and may be the mechanism involved here. The organic matrix has been shown (Puverel et al., 2005) to contain proteins with 36.5% and 45.5% of the amino acids present as aspartic and glutamic acid respectively. One of two sequences of *P pistillata* included a long poly-aspartate domain with 36 aspartic residues. While Puverel et al. (2005) suggested a calcium binding function it is also possible that these amphoteric amino acids may have a role in the sequestration of protons. If protons are sequestered for any length of time TA may be affected. How sufficient calcium ions get into the calcicoblastic layer is not clear, but toxins produced by zooxanthellae may open Ca^{2+} channels (McConnaughey, 2012) or, as suggested by Sandeman (2008b) hydrogen peroxide generated during photosynthesis might make the plasma membrane leaky to calcium ions.

The experiments with low and increased illumination indicated that mean calcification rate increased from $0.063\text{mg}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ to

Live coral

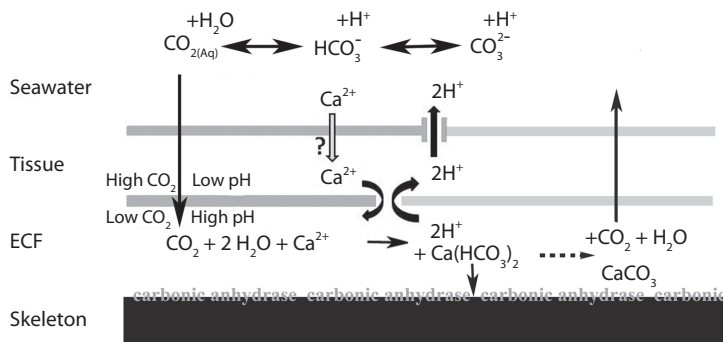


Fig. 5. Schematic model for calcification. CO_2 is uncharged and diffuses through the tissues to the ECF. Ca^{2+} is actively transported into the ECF by the Ca^{2+} ATPase pump and protons are transported in the opposite direction. The latter enhances diffusion of CO_2 into the ECF. Protons produced in the ECF are transported into tissue layers by the Ca^{2+} ATPase pump, from there the protons are probably transferred out of cells by voltage gated channels. The organic matrix has calcium binding properties and carbonic anhydrase which catalyses the reaction in which $\text{Ca}(\text{HCO}_3)_2$ is formed on the surfaces in the organic matrix. The $\text{Ca}(\text{HCO}_3)_2$ later slowly converts (dotted arrow) to CaCO_3 releasing CO_2 which can diffuse out or be re-combined with water to form more $\text{Ca}(\text{HCO}_3)_2$.

0.098 mg.hr⁻¹.cm⁻² with increased illumination which is as expected. The rate of change of pH in the seawater medium was from -0.0076 pH units.hr⁻¹.cm⁻² to -0.0030 pH units.hr⁻¹.cm⁻² in increased illumination. A reduction of pH in the medium is the result of transfer of CO₂ into it, and it has to be from the coral. It is to be expected that if CO₂ is being taken up by zooxanthellae and used for photosynthesis that the amount transferred would be less. A change of -0.0076 in pH of the seawater would result from an increase of 4.9 μmol of CO₂ and -0.0030 from an increase of 1.92 μmol of CO₂, so this would indicate an uptake of 3 μmol of CO₂ by the algae.

Calcium bicarbonate is known to only exist in solution so it may seem an unlikely candidate for involvement in calcification. Experimentally, solutions of calcium bicarbonate (Ca²⁺, 320-370 ppm) will precipitate CaCO₃ while slowly releasing CO₂. If magnesium ions are present calcite formation is inhibited and aragonite precipitation favoured (Kitano et al., 1979). It seems possible therefore that if the right ions are present, a fairly concentrated solution of calcium bicarbonate could build up in the spaces of the organic matrix and slowly convert to a deposit of aragonite on the surface present. The unusual results obtained in this study are explainable by an initial formation of calcium bicarbonate and its slow conversion to carbonate. The temporal separation between uptake of Ca²⁺ (change of TA) and movements in, then later out, of CO₂ (changes in DIC), means that care should be applied in the application of the alkalinity anomaly technique for the measurement of calcification in corals.

RESUMEN

Resultados inesperados de medición directa, con una microbalanza de torsión en un sistema cerrado, de las tasas de calcificación de los corales *Agaricia agaricites* (Scleractinia:Agariciidae) y concomitantes cambios de pH en el medio del mar. La acidificación del océano está impactando la calcificación de los corales, pero los mecanismos de la calcificación son aún inciertos. Para explorar la relación entre la calcificación y pH, pequeños trozos de coral fueron suspendidos en una microbalanza de

torsión en agitado suave, temperatura controlada, y agua de mar en una cámara cerrada. La tasa de calcificación neta y el pH se monitorearon continuamente mientras que la luz, temperatura o pH podían ser manipulados. Las piezas de coral eran de los bordes de placas finas de *Agaricia agaricites* y se estudiaron vivos y recién colectados. Inesperadamente, cuando la calcificación (n= 9, 0.082 mg.hr⁻¹.cm⁻²) se estaba dando, según lo determinado por el aumento de peso, el pH del agua de mar circundante cambió poco (n = 10, -0.0047 pH units.hr⁻¹.cm⁻²). Durante los períodos cuando la calcificación no se estaba dando la disminución del pH del agua de mar era un orden de magnitud mayor, -0.013 pH units.hr⁻¹.cm⁻². Esto es exactamente lo contrario de lo que se espera cuando se forma carbonato de calcio (CaCO₃). Del mismo modo un esqueleto recién colectado al inicio no mostró cambios de pH en el agua de mar aunque eran muy altas las tasas de ganancia de peso (hasta 1.0 mg hr⁻¹.cm⁻²). Después de 10 horas, la tasa de deposición disminuyó hasta seguir una curva de crecimiento generalizada de Michaelis-Menten, el pH comenzó a disminuir drásticamente, lo que indica un aumento de CO₂ en el agua de mar. Estos resultados inesperados pueden explicarse si el bicarbonato de calcio inestable (Ca(HCO₃)₂) se forma en la superficie de la anhidrasa carbónica/matriz orgánica y lentamente se transforma más tarde a CaCO₃. Piezas de coral vivo vigiladas en la cámara durante 30 horas demostraron un patrón de ganancia de peso durante el día y de pérdida en la noche. La pérdida sería coherente con la transformación de la Ca (HCO₃)₂ a CaCO₃ con el lanzamiento de CO₂. La tasa de calcificación media de coral vivo fue mayor (n= 8, p= 0.0027) en luz alta (120 μmol.s⁻¹.m⁻²) a 0.098 mg.hr⁻¹.cm⁻², en comparación con 0.063 mg.hr⁻¹.cm⁻² en condiciones de poca luz (12 μmol.s⁻¹.m⁻²). Sin embargo, al mismo tiempo la tasa media de cambio de pH fue de -0.0076 bajo luz baja en comparación con -0.0030 bajo luz alta (n= 8, p= 0,0001). La diferencia puede explicarse porque el CO₂ está siendo utilizado para la fotosíntesis por zooxantelas. La tasa de deposición de coral vivo no fue afectada por la adición de fosfato pero la tasa de ganancia de peso de los esqueletos recién colectados era fuertemente reforzada por fosfato. Estos resultados indican que la atención debe aplicarse en la aplicación de la técnica de alcalinidad anormal para la medición de la calcificación de los corales.

Palabras clave: coral, calcificación, CO₂, pH, matriz orgánica, anhidrasa carbónica, Ca (HCO₃)₂.

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