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Measuring gross and net calcification of a reef coral under ocean acidification conditions: methodological considerations

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Abstract

Ongoing ocean acidification (OA) is rapidly altering carbonate chemistry in the oceans. The projected changes will likely have deleterious consequences for coral reefs by negatively affecting their growth. Nonetheless, diverse responses of reef-building corals calcification to OA hinder our ability to decipher reef susceptibility to elevated pCO_2. Some of the inconsistencies between studies originate in measuring net calcification (NC), which does not always consider the proportions of the "real" (gross) calcification (GC) and gross dissolution in the observed response. Here we show that microcolonies of *Stylophora pistillata* (entirely covered by tissue), incubated under normal (8.2) and reduced (7.6) pH conditions for 16 months, survived and added new skeletal $CaCO_3$, despite low (1.25) Ω_{arg} conditions. Moreover, corals maintained their NC and GC rates under reduced (7.6) pH conditions and displayed positive NC rates at the low-end (7.3) pH treatment while bare coral skeleton underwent marked dissolution. Our findings suggest that *S. pistillata* may fall into the "low sensitivity" group with respect to OA and that their overlying tissue may be a key determinant in setting their tolerance to reduced pH by limiting dissolution and allowing them to calcify. This study is the first to measure GC and NC rates for a tropical scleractinian corals under OA conditions. We provide a detailed, realistic assessment of the problematic nature of previously accepted methods for measuring calcification (total alkalinity and ^{45}Ca).

1 Introduction

Coral reefs are being threatened by climate change (e.g. McNeil, 2004; Solomon, 2007; Hoegh-Guldberg et al., 2007) and ocean acidification (Kleypas et al., 2006; Hoegh-Guldberg et al., 2007; Guinotte and Fabry, 2008; Kleypas and Yates, 2009). The latter process is currently altering seawater carbonate chemistry, namely an increase in dissolved inorganic carbon (DIC) and pCO_2, reduction in aragonite saturation state and pH (Kleypas et al., 1999; Zeebe and Wolf-Gladrow, 2001) with projections for

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a decrease of 0.3–0.4 pH units by the end of this century (Caldeira and Wickett, 2005; Orr et al., 2005). Mounting experimental evidence suggests that net calcification rates of adult reef-building corals may decrease by 12–56 % under doubled preindustrial CO_2 concentration (Table 1; e.g. Schneider and Erez, 2006; Jokiel et al., 2008) and may negatively affect the settlement and post-settlement calcification and growth of early life stages of corals (Table 1; e.g. Albright et al., 2010). Slower growth rates might have detrimental implications for the ability of coral reefs to maintain a positive balance between reef accretion and reef erosion (Kleypas et al., 2006; Hoegh-Guldberg et al., 2007; Guinotte and Fabry, 2008). Nonetheless, recent studies show that some tropical scleractinian corals may calcify at normal or even higher rates, under ocean acidification (OA) conditions (Table 1; Reynaud et al., 2003; Jury et al., 2010; Anthony et al., 2008; Krief et al., 2010), suggesting that coral calcification response to OA may be more complex than previously thought with distinct species-specific sensitivity.

As the aforementioned studies measured the net calcification (NC; gross calcification minus dissolution) of corals, without discriminating calcification from skeleton dissolution (Kleypas et al., 2006; Riebesell et al., 2010; Rodolfo-Metalpa et al., 2011), it is difficult to determine whether the apparent decrease in calcification, recorded in those studies, is due primarily to a decrease in the "true" calcification (gross calcification), increased skeleton dissolution (gross dissolution) or a combination of both (net calcification; Rodolfo-Metalpa et al., 2011).

The most commonly used methods for measuring net calcification (= gross calcification-dissolution) in living corals are the buoyant weight and total alkalinity (Riebesell et al., 2010). The radio-isotope ^{45}Ca labeling technique is the only one likely to provide measurements of gross calcification (GC) in short-term incubation experiments.

While in the total alkalinity method NC is measured by tracking changes in TA in the seawater surrounding the incubated corals (Chisholm and Gattuso, 1991), in the radioactive technique GC values are obtained by measuring the incorporation of ^{45}Ca to the coral skeleton (Tambutte et al., 1995). Using the radioactive isotope method for

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investigating coral calcification involves some shortcomings (Buddemeier and Kinzie, 1976) with the major obstacle being an isotopic exchange between the ^{45}Ca in the medium and the non-radioactive calcium at the surface of the coral skeleton, resulting in an apparent calcification in the absence of biological activity (Goreau and Goreau, 1960; Clausen and Roth, 1975; Barnes and Crossland, 1977). In the present study, an improved ^{45}Ca protocol (Tambutte et al., 1995) was employed using cultured microcolonies (Almoghrabi et al., 1993) entirely covered by coral tissue which prevented non-specific ^{45}Ca binding with the skeleton.

To the best of our knowledge, Smith and Roth (in Smith and Kinsey, 1978) and Tambutte et al. (1995) are the only studies to compare the total alkalinity anomaly and ^{45}Ca incorporation techniques for corals. Both studies had similar results and found a high correlation between the two methods but did not compare them under varying carbonate chemistry conditions. Rodolfo-Metalpa et al. (2011) used the total alkalinity and ^{45}Ca techniques to determine the effect of ocean acidification (OA) conditions on the NC and GC of Mediterranean calcifying organisms. In that study, the importance of the organism tissue for internal pH management was highlighted. Of the four single-cell thick epithelial layers (oral ectoderm and endoderm and the aboral ectoderm and endoderm) interconnected by the thin non-cellular mesoglea, only the oral ecotoderm is in direct contact with seawater. It is most acceptable that calcifying organisms' tissue forms a barrier isolating the calcifying fluid from the external seawater (e.g. Goreau, 1959; McConnaughey and Whelan, 1997; Kleypas et al., 2006; McConnaughey and Gillikin, 2008) and have the ability to raise the pH at the site of calcification (Kuhl et al., 1995; Furla et al., 1998; Al-Horani et al., 2003; Cohen and McConnaughey, 2003; Cohen and Holcomb, 2009; Cohen et al., 2009; Ries, 2011). This ability of the organism tissue to elevate internal pH may allow calcification and prevent dissolution in acidified seawater.

In the present study we investigated the role of coral tissue in determining the vulnerability of the reef-building coral *Stylophora pistillata* to OA (pH_T 7.19 and 7.49) by measuring its NC, GC and dissolution rates. We compared (1) NC of intact corals with

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dissolution rates of bare coral skeletons, and (2) NC with GC of intact corals after long-term (14 months) exposure to reduced pH conditions. We also compared results obtained using the two aforementioned methods and discuss their suitability for measurements of calcification under high pCO_2 conditions.

2 Materials and methods

2.1 Coral preparation and maintenance

Eight colonies of the scleractinian coral *Sylophora pistillata* were collected by scuba diving from a depth of 4–8 m near the Interuniversity Institute for Marine Sciences (IUI) in Eilat, Israel (29°30′ N 34°55′ E). Following fragmentation, pieces (3–5 cm long) were suspended on nylon thread allowing the tissue to grow over the exposed skeleton. In addition to being entirely covered by tissue, fragments used in the ^{45}Ca procedure and the intercomparison study (TA vs. ^{45}Ca) had similar shape, size (2.5–3 cm) and same genotype (hereafter microcolonies; Almoghrabi et al., 1993; Tambutte et al., 1995). Moreover, microcolonies were free of boring organisms, minimizing the biological variation between specimens. Corals were maintained in water tables (150 l) with a flow-through system supplied with seawater from a depth of 30 m. The incoming seawater presented a very stable chemistry during the experiment: salinity of 40 ± 0.2 ‰, pH_T of 8.190 ± 0.017, total alkalinity of 2505 ± 5 µeq kg^{-1} (Fig. S1), as reported by the Israel National Monitoring Program (NMP) of the Gulf of Eilat. Temperature was regulated to ~ 25 °C using a combination of an array of 300 W BluClima aquarium heaters (Ferplast Spa, Vicenza, Italy) and an air-conditioner. Light (~ 170 µmol photons m^{-2} s^{-1}, 10 l: 14D photoperiod) was provided by a metal halide lamp (14 000 K, 400 W/D, Osram GmBH, Germany) and measured by a quantum irradiance meter (LiCor). Water motion was generated by power heads. Corals were fed once a week with freshly hatched *Artemia naupli* and a mixture of crushed fish. After a one month recovery period, coral skeletons were entirely covered by tissue.

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2.2 Measurements of gross and net calcification

2.2.1 Incubation procedure

Fragments of *S. pistillata* or microcolonies were incubated for up to 6 h in vessels containing filtered seawater (FSW; 0.45 µm membranes) at two pH treatments: 8.09 (pCO_2 = 387 µatm; ambient) and 7.49 (pCO_2 = 1917 µatm) or 7.19 (pCO_2 = 3898 µatm; Table 2) Corals in the low-end pH treatment were exposed to substantially high pCO_2 (approximately six times the predicted CO_2 level by 2100 according to Caldeira and Wickett, 2005) to investigate the physiological response at $\Omega_{arg} < 1$. To avoid confusion, we use the term "acclimation" in this paper to indicate long-term incubation at a certain pH condition. Temperature and light intensity conditions were similar to those provided during the culture period. A shaking water bath provided both water motion and constant temperature (Table 3). The microcolonies were rapidly transferred to the vessels and capped. Prior to the incubation period, the colony surface and attached nylon strings were carefully cleaned of epiphytes and algae.

2.2.2 Net calcification (NC)

Net calcification rates of corals incubated under light and dark conditions were compared to dissolution rates of fragments lacking tissue. For this, samples were immersed in sodium hypochlorite (NaOCl) overnight and subsequently rinsed thoroughly with DDW. pH was measured and water samples were collected for alkalinity at the beginning and end of the incubation from each treatment to determine carbonate chemistry. Water samples were stored in the dark at 4 °C until analyzed. Calcification rates were calculated from the difference between TA measured at the beginning and the end of each incubation period according to the equation by Schneider and Erez (2006). The same equation was used to measure dissolution of bare coral skeleton.

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2.2.3 Gross calcification (GC)

Microcolonies were placed in 40 ml incubation vessels containing FSW with a total activity of 360 kBq (^{45}Ca as $CaCl_2$, 1958.18 MBq ml^{-1}, PerkinElmer Life and Analytical Sciences). Dead specimens killed with 2 % formaldehyde were included in the experiment as a control for isotopic exchange (Al-Horani et al., 2005). 100-µl aliquots were taken at the beginning and end of each incubation to determine the specific activity. Following the labeling period, specimens were immersed in 600 ml FSW for 1 min, and then rinsed five times (each rinse lasting 1 min) with 10 ml of ice-cold glycine-high calcium medium (50 mM $CaCl_2$, 950 mM glycine, pH adjusted to 8.2). Labeled specimens were then incubated for 30 min in vessels containing 20 ml of ^{45}Ca-free sea water. Water motion was provided by a shaker. Following efflux incubation, microcolonies tissue was removed using 2 M NaOH for 20 min at 90 °C. After tissue hydrolysis, the skeleton was first rinsed with 1 ml NaOH (Houlbreque et al., 2003) then thoroughly rinsed with FSW and finally with DDW (Tambutte et al., 1995). The solution from the first rinse was added to the tissue hydrolysate and the remaining of the rinsing solution was decanted. Finally, skeletons were dried at 70 °C and subsequently dissolved in 12 M HCl. Samples (500 µl) of skeleton digest and tissue hydrolysate were added to 10 ml Ultima Gold AB (PerkinElmer) scintillation liquid and measured on a scintillation counter (Tri-carb 1600TR, Packard). Calcification rates were then calculated from the activity recorded in seawater control samples and was given in µmol Ca^{2+} per skeleton dry weight (Houlbreque et al., 2003; Tambutte et al., 1996).

2.2.4 Comparing gross and net calcification

The alkalinity and ^{45}Ca experiment were carried out over four days, with two consecutive days per pH treatment, using the same microcolonies, the same experimental design (as described previously; Table 3) and at an equivalent time of day to avoid any error that might be caused by diurnal variations (Edmunds and Spencer-Davies, 1988). NC and GC were calculated and evaluated by comparing between their mean values

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for each time point and determining the correlation between both measurements type. Prior to the experiment, the respective microcolonies were maintained in pH treatments (8.09 and 7.49 on the pH_T scale) for a period of 14 month.

2.3 Calculation of carbonate system in seawater

Total alkalinity (TA) values were measured using an automatic potentiometric titration to the second end point (Almgren et al., 1983). It was then computed using the Gran equation (DOE, 1994) or the first derivative of the curve for the evaluation of the exact end point (see Supplement). Components of the carbonate system (pCO_2, CO_3^{-2}, HCO_3, DIC concentrations and Ω aragonite) were calculated from total alkalinity along with pH values (see Supplement), temperature and salinity using the CO2SYS program, version 01.03 (Lewis and Wallace, 1998; Table 2). The pH_{NBS} were shifted onto the total pH scale (pH_T) by subtracting −0.11 (Zeebe and 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 ‰.

2.4 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. 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), which monitored the pH and controlled the bubbling of CO_2 (from a CO_2 cylinder) to each tank according to the desired pH. Well-mixed water from each tank continuously flowed into the corresponding section (150 l) of the water table. All pH data were recorded using monitoring software (Timo, Matuta, Germany; 7.21 ± 0.2, 7.45 ± 0.2 and 8.09 ± 0.01 for pH_T 7.19, 7.49 and 8.09, respectively). Seawater samples from the pH system were measured for total alkalinity to monitor the system numerous times over the experimental time period (2501 ± 13, 2491 ± 9 and 2501 ± 6 µeq k^{-1} for pH_T 7.19, 7.49 and 8.09, respectively; Fig. S1).

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2.5 Normalization measurements

Surface area was measured utilizing one of the common methods currently used, the paraffin wax method (Stimson and Kinzie III, 1991). Coral volume was determined by measuring its displacement in seawater in two different ways. Skeleton dry weight was determined using an analytical balance.

2.6 Statistical analysis

Details of statistical tests performed and all the results are in the Supplement; analyses were performed using the statistical software SPSS 15 (for standard one- or two-way ANOVA) and R software version 2.13.2 (for ANOVA permutation test, Akaike's Information Criterion and Reduced Major Axis regression; R Development Core Team, 2006).

3 Results

In general, corals that were incubated in the light and at the ambient pH calcified at rates ($0.146 \pm 0.05\,\mu mol\,CaCO_3\,cm^{-2}\,h^{-1}$) similar to those observed by Schneider and Erez (2006), who also used *S. pistillata* collected in the same study site as the present study.

3.1 Comparison of light, dark and coral skeleton treatments

Net calcification rates were compared between corals that were not exposed to reduced pH treatments prior to incubation (pH_T 7.19) and corals that were exposed for two, five and fourteen months (pH_T 7.49; referring only to the 2 h of incubation results; Table 3; Fig. 1). Model selection by AIC_c indicated that the global model, the combination of pH, treatment and experiment type is unambiguously the best-fit model (analyzed with permutation ANOVA test) to explain variation in calcification rates, with over 99 %

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confidence (Akaike weight). When net calcification/dissolution was measured for light and dark-incubated corals and corals deprived of tissue, the rates of NC were treatment specific (TukeyHSD, $p < 0.001$; Fig. 1) independent of pH (8.09, 7.19 and 7.49 on the pH_T scale; TukeyHSD, $p = 0.02$). Dark calcification obtained from corals exposed for two months to pH_T 7.49 differed significantly when compared to those obtained from corals exposed for five months to the same pH treatment ($p = 0.04$). Corals under dark conditions that were not exposed to reduced pH treatment (pH_T 7.19) and those which were exposed for two months prior to incubation (pH_T 7.49) exhibited net dissolution of coral skeleton. Bare coral skeletons started to dissolve under pH_T 7.19 treatment whereas skeletons treated at pH_T 8.09 and 7.49 displayed values closer to zero (alkalinity results were in the range of instrument limitation, 60 µeqv kg^{-1}; $p < 0.001$).

3.2 Comparing gross and net calcification

The strength of relationship between estimates of calcification rates measured by the alkalinity and the ^{45}Ca techniques was computed for each pH treatment separately (Fig. 2c) using the RMA regression analysis. At ambient pH, both methods were correlated with a correlation coefficient (r) of 0.872 ($p < 0.001$). The relationship at the low pH treatment was less correlated ($r = 0.561$, $p = 0.015$) and did not follow the 1 : 1 regression line as indicated by the regression slope.

When mean values of total calcification were compared, no significant differences were recorded between methods (permutation test for repeated measure ANOVA, $p = 0.171$; Fig. 2a, b; Tables S1, S2). Total calcification values as obtained from both methods were lower at pH_T 8.09 as compared to pH_T 7.49 after 4 h of incubation only (58 % and 68 % based on the alkalinity technique and ^{45}Ca method, respectively; Tukey-adjusted, $p = 0.017$) suggesting that the measured trend is genuine and not a methodological bias. In addition, at pH_T 7.49 total calcification varied with time: values were lower after 2 h of incubation as compared to 4 and 6 h of incubation. This suggests an apparent acceleration of calcification rates over time.

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Methods differed significantly in their precision (permutation test for repeated measure ANOVA; $p = 0.003$) showing different levels of variation in the calcification measurements at the low (7.49) pH_T treatment after 6 h of incubation (Tukey-adjusted, $p = 0.032$). These findings were also inferred from the results of the Levene's test of homogeneity of variance.

4 Discussion

4.1 Comparing NC of living corals with the dissolution of dead samples

Separating NC rates of intact corals from dissolution rates of bare coral skeleton was done to examine whether the presence of the tissue has an effect on the dissolution of coral skeletons. Corals under light, at pH_T 7.19 and 7.49, were able to maintain positive calcification values although bare coral skeletons exhibited dissolution. The latter are in agreement with Rodolfo-Metalapa et al. (2011) who reported on the dissolution of *Balanophyllia europaea* lacking tissue at a pH_T of 7.4 as compared to positive NC of coral specimens that were completely covered by tissue. These authors pointed out that the lack of external overlying tissue in the dead samples caused relatively high dissolution rates at this undersaturated condition ($\Omega_{arg} < 1$). It is likely, that the tissue of *S. pistillata* in our study limited dissolution of coral skeleton under acidification conditions. Moreover, NC of light-incubated corals as compared with the net dissolution of dark-incubated corals at low pH treatment (corals exposed to pH_T 7.19 with no acclimation period and corals acclimated for 2 months at pH_T 7.49; Fig. 1 (demonstrate that corals can regulate calcification through changes in internal conditions (e.g. internal pH and Ω_{arg}; Goreau, 1959; Kuhl et al., 1995; Krief et al., 2010; McConnaughey and Whelan, 1997; Furla et al., 1998; Al-Horani et al., 2003; Ries, 2011) associated with biological activities of the living tissue and consequently creating conditions that may favor the precipitation or dissolution of calcium carbonate. Differences in ΔpH measured in the external medium and coral calcification rates at both light and dark

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incubations (e.g. increase in pH caused by photosynthesis; results not shown) may also indicate that these co-occurring biological processes alter conditions in the surrounding seawater. Our results demonstrate a higher rate of dark calcification in the 5 months acclimated corals as compared with corals that were incubated for only 2 months. While this can be attributed to the low pH (~ 7.21 on the pH_T scale; $\Omega_{arg} < 1$) measured in the vessels containing the 2 months incubated corals, it is unclear at this stage if acclimation/incubation period has an effect on the examined species dark calcification potential. These light and dark differences in pH and calcification may reinforce the importance of the overlying tissue. In this kind of experiment, however, we cannot distinguish between the true rate of calcification and dissolution because alkalinity measures net values only.

4.2 Calcification response to high pCO_2

Most published studies to date report negative effects to varying degrees on the calcification rates of reef-building corals as a result of seawater acidification (Table 1; e.g. Schneider and Erez, 2006; Jokiel et al., 2008) while others indicate insensitivities of some corals to OA (Reynaud et al., 2003; Anthony et al., 2008; Jokiel et al., 2008; Jury et al., 2010). Following 16 months of incubation under elevated pCO_2 conditions (pH_T 7.49), all individual coral fragments in the current study survived and added new skeletal $CaCO_3$, despite Ω_{arg} values as low as 1.25. Furthermore, our results of light incubation showed no significant differences in corals NC between ambient (pH_T 8.09) and reduced (pH_T 7.19 or 7.49) pH conditions (Fig. 1). These results were consistent over all experiments regardless of experimental design, acclimation time to the reduced pH treatment or colony genotype. Although dark-incubated corals exhibited net dissolution at the reduced pH treatment (corals exposed to pH_T 7.19 with no acclimation period and corals acclimated for 2 months at pH_T 7.49; Fig. 1), no differences were found between calculated daily calcification rates of both pH treatments (12-h light-dark cycle, assuming that recorded light and dark calcification can represent the average day and night calcification; Moya et al., 2008). We attribute these findings mainly to the

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large variability in coral calcification response in each treatment (corals exposed to pH_T 7.19 with no acclimation period: 1.523 ± 0.438 and 1.194 ± 0.342 µmol $CaCO_3$ cm^{-2} d^{-1} for pH_T 8.09 and 7.49, respectively; corals acclimated for 2 months at pH_T 7.49: 2.824 ± 0.973 and 2.023 ± 1.215 µmol $CaCO_3$ cm^{-2} d^{-1} for pH_T 8.09 and 7.49, respectively).

Some of the contradictory responses in coral calcification between the studies outlined above may be related to differences in the methods used to estimate calcification (e.g. buoyant weight vs. alkalinity), experimental design (e.g. single organism vs. multispecies mesocosms), length of experiments (days to years), sample size and/or species investigated (e.g. branching vs. massive). We point out that manipulation type carbonate chemistry (CO_2(gas) versus strong acid) is a major factor that could have caused differences between our study and previous research. Schulz et al. (2009) demonstrated considerable differences between both methods, in the HCO_3^- concentration at extreme pCO_2 ($\gg$700 µatm) levels. Provided that the hypothesis of HCO_3^- as a positive factor influencing coral calcification is true (Furla et al., 2000; Herfort et al., 2008; Marubini et al., 2008; Jury et al., 2010), differences in [HCO_3^-] may be enough to influence the magnitude of coral response (Schulz et al., 2009; Jury et al., 2010) at the low pH treatments used in the current experiment.

4.3 ^{45}Ca uptake vs. total alkalinity techniques: gross vs. net calcification

4.3.1 Standardization of calcification measurements for intercomparison studies

Similar to Tambutte et al. (1995), the strength of the relationship between methods was determined by using the RMA (geometric regression; Model II regression; Ricker, 1973) analysis, which is suitable when describing a relationship between two variables that are subjected to measurement errors and natural variability (Ricker, 1973; Jacques and Pilson, 1980). Smith and Kinsey (1978) and Tambutte et al. (1995) demonstrated a high correlation ($\sim$ 0.99) between the methods and displayed a similar slope ($\sim$ 0.87). In the

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present study, the relationship in the ambient pH was also significant with a slope of 1.09 and a correlation coefficient of 0.872 (Fig. 2c). Tambutte et al. (1995) also recorded higher calcification rates as obtained from the alkalinity-derived estimates as compared to the radioisotope estimates that they interpreted as loss of radioactivity during coral processing or to a time lag due to the ^{45}Ca loading of extracellular and tissue compartments. Such an offset between the methods should have been observed, in this study, within the first 2 h of incubation. However, no significant difference between both methods was found in the present study. At the low pH treatment, results were more complex. While no significant differences were found between mean of total calcification values of both methods, the strength of the relationship was weak (r = 0.561) and methods presented different levels of precision after 6 h of incubation. As can be seen from the graphs (Figs. 2c; S2), the samples' standard deviations at 4 and 6 h of incubation were strongly distorted by outliers derived from the alkalinity estimates. This suggested considerable discrepancies between the recorded measurements. Provided that differences between GC and NC would have occurred (e.g. dissolution of coral skeleton will lower the NC whereas GC remains the same) it could have affected mean values and the precision level of both methods. These however should have been detected already after 2 h of incubation.

Substantial alteration in pH (Tables S3 and S4) and O_2 (results not shown) concentrations that were recorded inside the incubation vessels after 4 and 6 h of incubation may shed some light on the large variation between methods at the low pH treatment. pH and [O_2] changes may also explain the inconsistencies in coral calcification response between this study and previous ones. Biological processes in the coral holobiont (e.g. photosynthesis, respiration and calcification) can severely distort the initial values of the carbonate chemistry parameters (TA, DIC, pH, pCO_2, HCO_3^-, CO_3^{2-} and Ω_{arg}), especially in a closed system. Note that CO_2-enriched seawater has a lower buffering capacity (than normal seawater) resulting in a stronger drift in pCO_2 and pH in response to biological activity (Suzuki, 1998; Delille et al., 2005; Riebesell et al., 2007). Several studies have discussed the importance of choosing a suitable

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incubation volume (relative to coral biomass) and experimental duration so that the changes in the carbonate chemistry parameters will be relatively small as compared to the difference in those parameters between treatments (e.g. Chisholm and Gattuso, 1991; Leclercq et al., 2000; Langdon and Atkinson, 2005; Schulz et al., 2009; Riebesell et al., 2010). While a certain amount of change is needed to obtain accurate measurements of calcification, it is recommended that changes in TA and DIC should be less than 10 % (Schulz et al., 2009; Jury et al., 2010; Riebesell et al., 2010). A ∼ 20 % decrease in TA, however, was shown to have no effect on the rate of community calcification (Leclercq et al., 2000). In the present study, substantial differences in the concentrations of all carbonate system parameters were recorded at the end of the incubation at both pH treatments, exceeding +300 % change from initial conditions at pH_T 7.49, for CO_3^{2-} ions following 6 h of incubation (Tables S3 and S4). These large differences may in turn modify coral calcification response and disguise their sensitivity to OA. It should be pointed out that although TA and DIC after 2-h incubation were in the recommended percentage changes, the difference from initial condition in the $[CO_3^{2-}]$ (24 % and 73 % for pH_T 8.09 and 7.49, respectively) perhaps may have been large enough to affect coral response as CO_3^{2-} ion is considered to play a key role in coral calcification (e.g. Leclercq et al., 2000; Schneider and Erez, 2006; Cohen and Holcomb, 2009). Only minor variations in the carbonate parameters occur on coral reefs where the daily average values vary between 2457–2494 µeqv kg^{-1} for total alkalinity and between 8.16–8.33 for pH (Silverman et al., 2007b,a). pH variations also have the potential to affect the speciation of major elements (pH-dependent) in the system besides carbonate species (Zirino and Yamamoto, 1972), influence cellular metabolism processes that are pH sensitive (Cubells et al., 1994) and cause a shift in the microbial community associated with the coral (Thurber et al., 2009). High levels of O_2 concentrations recorded between two and 6 h of incubation (200–300 % supersaturation; data not shown) may have limited the photosynthesis process through photorespiration (Jordan and Ogren, 1981; Mass et al., 2010) in addition to the production and accumulation

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of reactive oxygen species (ROS; Lesser, 1996), which in turn may have increased the level of stress in the coral.

It is also possible that part of the variation observed is due to the natural variation in the calcification of a certain coral specimen and/or differences in measurement type (TA measures the water and ^{45}Ca measures the skeleton). Naturally, increasing the number of samples in the experimental design could also have helped in gaining more statistical power.

We concluded that at ambient pH, differences between the present study and the pervious intercomparison studies (Smith and Kinsey, 1978; Tambutte et al., 1995) can be attributed mainly to the fact that both measurement types (^{45}Ca and alkalinity) were conducted on separate days, which may have increased the error probability. It is possible to compare results of different studies in which only one of the two methods have been employed, as long as the experiments are conducted over very short-time spans and/or that the conditions are maintained constant. At the low pH treatment we failed to detect a strong compatibility between TA and ^{45}Ca estimates in addition to major changes in O_2 and carbonate system parameters that were found after 4 and 6 h of incubation. Additional intercomparison studies are therefore needed.

4.3.2 Effects of low pH treatment on the GC and NC of corals

In view of the vast changes in speciation of carbonate chemistry and the supersaturated conditions of O_2, NC and GC were compared only after 2 h of incubation. The similarity between NC and GC rates after 2 h of incubation suggested that the initial low Ω_{arg} (~ 1.25) conditions prevailing at pH_T 7.49 did not seem to cause any detectable dissolution of coral skeletons. Furthermore, although not significant, means of NC rates were higher than GC rates (0.634 ± 0.075 and 0.527 ± 0.091, respectively). It is possible however that the duration of incubation was too short for dissolution to take place or to have measurable dissolution rates. Acidification conditions did not seem to impair the "true" calcification of coral specimens as GC rates at both pH treatments were not significantly different. If at all the mean, mean of GC and NC rates at pH_T

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7.49 seemed slightly higher than those observed at pH_T 8.09 (it was significant only after 4 h of incubation). The same trend, although not significant, was also observed when NC rates of light-incubated corals were measured in corals that were exposed to the same pH treatment for two and five months. Rodolfo-Metalpa et al. (2011) showed that some temperate corals and mollusks were able to calcify and grow (GC) at even faster than normal rates when exposed to elevated pCO_2. Rodolfo-Metalpa et al. (2011) suggested that the external organic layer protects the shell or skeleton of marine calcifiers from dissolution under corrosive conditions and allows them to grow. This may be a key in determining their relative susceptibility to OA conditions (Ries et al., 2009; Rodolfo-Metalpa et al., 2011). In a previous study, Ries et al. (2009) briefly discussed the tolerance of different calcifying species (e.g. mollusk, corals, algae) to OA whose shell or skeleton were completely covered by tissue.

Our findings indicate that *S. pistillata* will be able to acclimate and even maintain normal calcification rates in a high CO_2 world even if dissolution will occur during nighttime, which implies that *S. pistillata* may fall into the CO_2-tolerant group. Coral ability to maintain their NC and GC rates under pH_T 7.49, along with the fact that bare coral skeleton underwent marked dissolution at pH_T 7.19 as compared with the positive NC rates of fully covered corals, may support our theory that *S. pistillata* tissue coverage protects the skeleton from dissolution and allows the coral to calcify under acidification conditions, thus play a role in determining *S. pistillata* tolerance to OA. It is likely that undersaturated conditions will increase dissolution of other coral species if their skeleton is partially exposed (e.g. *Cladocora caespitosa*) hence increasing their sensitivity to anticipated changes in CO_2.

Supplementary material related to this article is available online at: http://www.biogeosciences-discuss.net/9/8241/2012/bgd-9-8241-2012-supplement.pdf.

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Table 1. Changes in reef coral calcification rates in response to increased pCO_2 concentrations (relative to preindustrial concentration). s, settlement; ps, post settlement.

Organism/System	Approx. % change in calcification when pCO_2 is 2× preindustrial	Approx. % change in calcification when pCO_2 is 3× preindustrial	References	Notes
Scleractinian corals				
Stylophora pistillata	0	−14	Gattuso et al. (1998)	
	+5–(−50)		Reynaud et al. (2003)	Level of response is temperature dependent
	0–(−15)	−21–(−30)	Marubini et al. (2008)	
Stylophora pistillata + *Porites* sp.		−18–(−75)	Krief et al. (2010)	
Acropora cervicornis		−66–(−76)	Renegar and Riegl (2005)	
Acropora eurystoma	−55		Schneider and Erez (2006)	
Acropora intermedia	−4–(−18)	−30–(−40)	Anthony et al. (2008)	Level of response is temperature dependent
Acropora verweyi	−18		Marubini et al. (2003)	
Porites compressa + *Montipora capitata*	−40–(−83)		Langdon and Atkinson (2005)	Level of response is season dependent
Porites compressa	higher than −10		Marubini et al. (2001)	
Porites lutea	−24–(−93)		Ohde and Hossian (2004)	
Porites lobata	+19–(−12)	not clear		
Porites australiensis		−7–(−10)	Iguchi at el. (2012)	compare to control
Porites spp.	0 and −17		Edmunds (2011)	Calcification normelized to tissue area and biomass, respectively
Galaxea Fascicularis	−16		Marubini et al. (2003)	
Pavona cactus	−18		Marubini et al. (2003)	
Turbinaria reniformis	−13		Marubini et al. (2003)	
Montipora capitata	−15–(−20)		Jokiel et al. (2008)	Coral nubbins
Madracis auretenra	0–(+16)		Jury et al. (2010)	
Mesocosm and field studies				
Biosphere 2	−40		Langdon et al. (2000)	Dominated by
	−85		Langdon et al. (2003)	
Monaco mesocosm	−21		Leclercq et al. (2000)	
	−14	−17	Leclercq et al. (2002)	
Mesocosm	−13 (coral assemblage) −70 (other calcifiers)		Andersson et al. (2009)	Calcification community dominated by the coral *Montipora*

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Table 1. Continued.

Organism/System	Approx. % change in calcification			References	Notes
	s/ps	when pCO_2 is 2× preindustrial	3× preindustrial		
Early life stages					
Porites astreoides	s	−42–(−45)	−55–(−60)	Albright and	Decrease in living
	ps	−16	−35	Langdon (2011)	specimens
	s	0	0	Albright et al. (2008)	Measurments of
	ps	−50	−78		lateral extention rates
	ps	−22 per 1.0 decrease in Ω_{arg}		de-Putron et al. (2011)	Weight measurment of primary corallite
Porites panamensis	s	0		Anlauf et al. (2011)	Measurments of dry
	ps	−3			skeleton weight
Favia fragum	ps	−37 per 1.0 decrease in Ω_{arg}			
	ps		−26	Cohen et al. (2009)	Average weight of skeletal elements
Acropora palmata	s	−45	−69	Albright et al. (2010)	Decrease in living
	ps	−39	−50		specimens
Acropora digitifera	ps		−25	Suwa et al. (2010)	After 7 days
Acropora tenuis	ps		−10		Compare to control

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Table 2. Seawater carbonate chemistry in the various pH treatments. Total alkalinity and pH were 1 measured while the inorganic carbon speciation and Ω_{arg} were calculated based on pH and alkalinity 2 measurements using the CO2SYS program (Lewis and Wallace, 1998; Pierrot et al., 2006).

pH total scale	TA ($\mu eqv\,kg^{-1}$)	DIC ($\mu mol\,kg^{-1}$)	pCO_2 (μatm)	$CO_{2(aq)}$ ($\mu mol\,kg^{-1}$)	HCO_3^- ($\mu mol\,kg^{-1}$)	CO_3^{2-} ($\mu mol\,kg^{-1}$)	Ω_{arg}
8.09	2501	2122	387	10.6	1846	265	4.02
7.49	2499	2431	1917	52	2295	82	1.25
7.19	2501	2544	3898	107.1	2393	44	0.67

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Table 3. Details of the experimental set-up. O, open; C, close; NC, net calcification; GC, gross calcification.

Pre-incubation	Incubation experiment						
Exposure period to pH treatment (months)	Open/closed vessels	Water motion	Fragment size (cm^2)/ volume ratio (ml)	Incubation period (h)	Initial pH_T	Repeats	Measurement type
No pre-inc.	O		0.35–0.45	3	8.09 and 7.19	7	NC
2	C	x	0.14–0.18	1	8.09 and 7.49	5	NC
5	C	x	0.07–0.1	1	8.09 and 7.49	9	NC
14	C	x	0.14–0.18	2, 4 and 6	8.09 and 7.49	6	NC and GC

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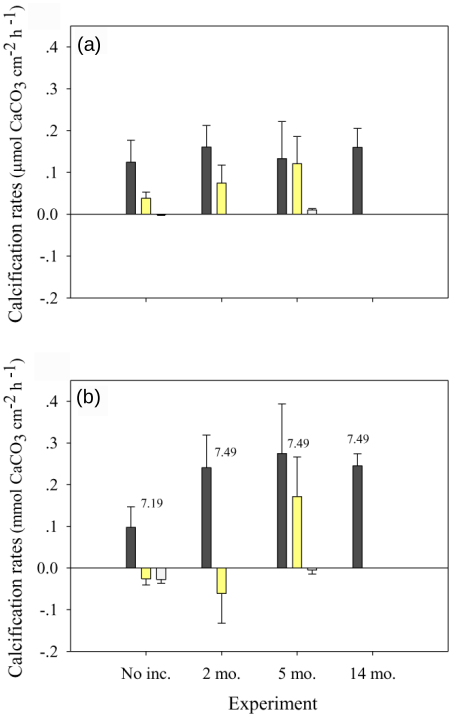

Fig. 1. Net calcification of *S. pistillata* fragments incubated under **(a)** normal (8.09) and **(b)** reduced (7.49 or 7.19) initial pH_T, as observed from corals that were not pre-exposed to pH treatments prior to incubation experiment (No inc.; pH_T 7.19; n = 3–7) and corals that were exposed to pH treatments for two (pH_T 7.49; n = 5), five (pH_T 7.49; n = 9) and fourteen (pH_T 7.49; n = 6; results are presented only for 2 h of incubation) months prior to incubation experiments. Specimens were incubated in light (grey bars) and dark (yellow bars) conditions and compared to fragments without tissue (white bars; mean ± STDV). mo., months.

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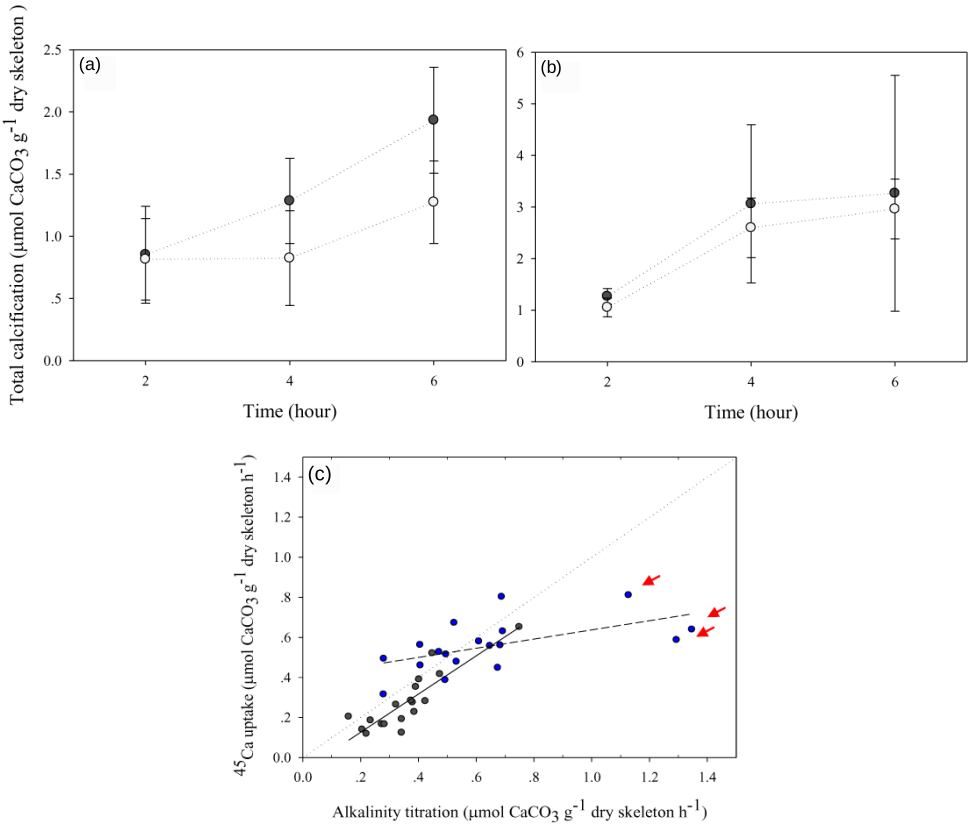

Fig. 2. Caption on next page.

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Fig. 2. Relationship between calcification values (total or rates) of *S. pistillata* microcolonies as estimated from total alkalinity (black circle) and ^{45}Ca incorporation (white circle) techniques. Microcolonies were incubated under normal (8.09) and reduced (7.49) initial pH_T and in the presence of light, over three time points, 2, 4 and 6 h; $n = 6$ for each time point. Microcolonies used in the experiment were cultured in the pH system for a period of 14 months before experiments were conducted. Calcification values of intact specimens, derived from the ^{45}Ca method, were corrected by subtracting ^{45}Ca uptake of the dead fra gments–control. Total calcification under 8.09 **(a)** and 7.49 **(b)** initial pH_T are presented as mean ± STDV of each time point; dark grey circles are the alkalinity technique; light grey circles are the ^{45}Ca technique. **(c)** Each point represents a measurement (GC or NC rates) for an individual coral. Linear regression analysis was used to examine relationships between both methods. Dotted line indicates 1 : 1 correlations. Red arrows indicate outliers. Data obtained by the two methods were correlated with a relationship described by the following equations: pH_T 8.09 (grey diamond), ^{45}Ca_uptake = 1.09 · TA-0114 (C.I., 0.81–1.38 and −0.215–(−0.013) for the slope and intercept, respectively), $r = 0.872$, $p < 0.001$; pH_T 7.49 (blue diamond), ^{45}Ca_uptake = 0.408 · Alkalinity + 0.292 (C.I., 0.229–0.587 and −0.215–(−0.013) for the slope and intercept, respectively), $r = 0.561$, $p = 0.015$.

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