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FEATURE ARTICLE

Comparison of the alkalinity and calcium anomaly techniques to estimate rates of net calcification

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ABSTRACT: Among current methods used to estimate calcification rates, the alkalinity anomaly technique has been widely used for short-term incubations as it is nondestructive and is based on a parameter that is easily and accurately measured. However, total alkalinity is also influenced by other processes such as nutrient consumption and release, and may also require corrections. The calcium anomaly technique has been little used because calcium is notoriously difficult to measure precisely and several species precipitate calcite with varying contents of magnesium. In this study, calcification rates of benthic calcifiers—a crustose coralline alga, a coral, a sea urchin, and a mussel—were estimated with these 2 approaches. Rates derived using the 2 methods were well correlated for all species and did not significantly differ from each other for corals incubated in the light and dark, and for coralline algae when incubated in the dark. Coralline algae appear to produce magnesian calcite in the light. Sea urchins and mussels released relatively large amounts of ammonia, and a correction of the alkalinity anomaly method was necessary. For urchins, calcification rates based on total alkalinity were higher than rates based on calcium as these organisms incorporate magnesium. For mussels, the corrected alkalinity anomaly technique provided values close to those based on calcium anomaly at low incubation densities, but values were significantly lower at high calcification and excretion rates, suggesting that ammonium concentrations were underestimated or another process affected total alkalinity. The results demonstrate that the alkalinity and calcium anomaly techniques are not robustly applicable to all calcifiers without careful consideration of organism physiology and mineralogy.
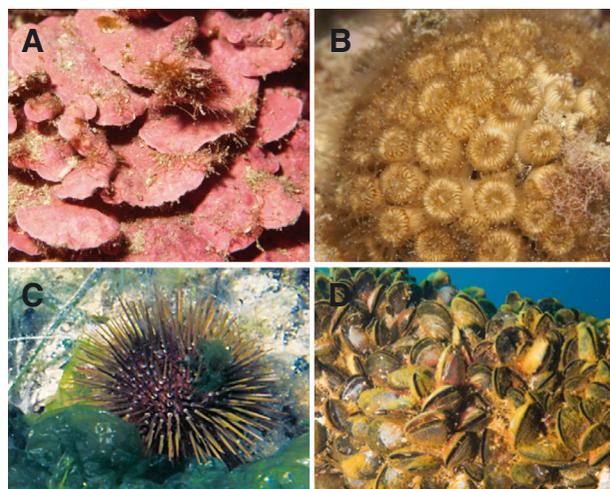
Calcification rates in 4 benthic calcifiers with distinct metabolism were determined by both the alkalinity and calcium anomaly techniques: (A) coralline alga, (B) scleractinian coral, (C) sea urchin, (D) mussel.

Photos: D. Luquet (OOV)

KEY WORDS: Calcification · Benthic species · Total alkalinity · Calcium · Methods

INTRODUCTION

Calcification is the biomineralization process by which some organisms produce skeletons or shells made of calcium carbonate ($CaCO_3$). It plays a major role in the global carbon cycle over different time scales (Milliman 1993) and is the key mechanism by which CO_2 is returned to the atmosphere (Berner et

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al. 1983). The capacity of marine organisms to produce $CaCO_3$ and its response to environmental drivers have been the subject of a growing body of research in the past decades. Indeed, oceanic carbonate chemistry is modified as a consequence of anthropogenic CO_2 emissions in the atmosphere and the absorption of a significant part of them by the ocean (25%; Le Quéré et al. 2009). Decreases in pH and carbonate ion concentrations ($[CO_3^{2-}]$) and increases in CO_2 and bicarbonate concentrations ($[HCO_3^-]$) have been observed; all these changes are collectively referred to as ocean acidification (Gattuso & Hansson 2011). Since calcification is believed to be dependent, to some extent, on environmental $[CO_3^{2-}]$ (Broecker & Takahashi 1966), ocean acidification often leads to decreased calcification (Kroeker et al. 2013) and may weaken the global carbonate counter-pump in the coming decades (Gehlen et al. 2007).

Several methods are available to quantify calcification rates of planktonic and benthic calcifiers. The buoyant weight technique consists of weighing an organism before and after a certain amount of time in seawater of a known density (calculated using salinity and temperature; Spencer Davies 1989). It assumes that soft tissues (including organics contained in the calcified structures) have a density close to that of seawater. It is nondestructive and offers the possibility of studying the same organism and following its calcification over time (e.g. Martin & Gattuso 2009, Bramanti et al. 2013, Gazeau et al. 2014). Another option is to estimate the linear extension (growth) of an organism, assuming the density of its calcifying structures is known. The use of this method seems to have been limited to zooxanthellate corals (Chalker et al. 1985). Changes in the concentration of particulate inorganic carbon (e.g. Riebesell et al. 2000) and calcium (e.g. Gazeau et al. 2011) as well as the incorporation of their radioactive isotopes ([45]Ca, Goreau & Bowen 1955; ^{14}C, Paasche 1963) in $CaCO_3$ have also been used. These techniques involve incubations in seawater with or without [45]Ca or [14]C. The organism must subsequently be sacrificed and the skeletal components dissolved in acid to measure C, Ca^{2+}, ^{14}C or [45]Ca. A very attractive alternative to these destructive methods is the quantification of the change in a seawater constituent that is closely related to calcification.

The alkalinity anomaly technique (Smith & Key 1975) has been widely used in short-term incubations or *in situ* studies to estimate net calcification of organisms and communities, especially of corals and coral reef environments. Total alkalinity (A_T) can be defined as the total buffering capacity of seawater, or the excess of proton acceptors over proton donors (Wolf-Gladrow et al. 2007):

$$A_T = [HCO_3^-] + 2[CO_3^{2-}] + [B(OH)_4^-] + [OH^-]$$
$$+ [HPO_4^{2-}] + 2[PO_4^{3-}] + [H_3SiO_4^-]$$
$$+ [NH_3] + [HS^-] - [HSO_4^-] - [H^+] - [HF]$$
$$- [H_3PO_4] + [\text{minor acids} - \text{minor bases}] \quad (1)$$

As can be seen in Eq. (1), A_T is highly influenced by $[HCO_3^-]$ and $[CO_3^{2-}]$ together with a multitude of other minor compounds. Calcification consumes carbonate or bicarbonate, following the reversible reaction:

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

Eq. (2) shows that calcification consumes 2 moles of HCO_3^-, therefore decreasing A_T by 2 moles per mole of $CaCO_3$ produced (see Eq. 1). It is possible to derive the rate of net calcification (gross calcification − dissolution) by measuring A_T before and after incubating an organism or a community. This method assumes, however, that only calcification influences A_T (Smith & Key 1975). Natural water samples contain various acid−base systems that can accept and donate protons (Wolf-Gladrow et al. 2007). For instance, nitrogen assimilation through photosynthetic activities has been experimentally shown to significantly impact A_T (Brewer & Goldman 1976). Aerobic and anaerobic (denitrification, sulphate reduction) remineralization of organic matter as well as nitrification are known to impact A_T through the consumption or release of nutrients (ammonium, nitrate and phosphate) and protons (Wolf-Gladrow et al. 2007). Although some potential limitations of the alkalinity anomaly technique were highlighted long ago (Chisholm & Barnes 1998, Smith & Key 1975), many different studies have made use of this method to estimate calcification rates of isolated organisms (e.g. zooxanthellate corals, Gattuso et al. 1998; mollusks, Gazeau et al. 2007; deep-sea corals, Maier et al. 2012; coralline algae, Martin et al. 2013) as well as of coral reefs (e.g. Gattuso et al. 1999).

In contrast to A_T, the concentration of calcium (Ca^{2+}) in seawater is only influenced by net calcification and a 1:1 relationship can be used to derive net calcification rates. The calcium anomaly technique has been used considerably less than the alkalinity anomaly method due to generally large measurement errors and large background calcium concentrations (~10.5 mmol kg^{-1} at a salinity of 35). However, methods based on automatic potentiometric titrations (Lebel & Poisson 1976) have the capability to precisely and accurately measure Ca^{2+} concentrations in seawater. Cao & Dai (2011) reported a preci-

sion of ca. 0.06%. As some marine calcifiers, e.g. echinoids and coralline algae, incorporate a variable but significant amount of magnesium (Mg^{2+}) in their calcite, the calcium anomaly technique will likely underestimate true calcification rates for these organisms.

Smith & Roth (cited in Smith & Kinsey 1978) and Tambutté et al. (1995) compared the alkalinity anomaly and the ^{45}Ca techniques for measuring the calcification rates of zooxanthellate corals. They reported a similar slope between the two but the intercepts had different signs, which Tambutté et al. (1995) attributed to specificities of their ^{45}Ca method. Chisholm & Gattuso (1991) and Murillo et al. (2014) found an excellent consistency of calcification rates measured by the alkalinity and calcium anomaly techniques for isolated zooxanthellate corals. Murillo et al. (2014) showed that the alkalinity anomaly technique provided rates that were significantly lower than those based on calcium when the whole community, including sediment, was incubated.

The objective of the present study is to evaluate the capacity of the alkalinity and calcium anomaly techniques to accurately measure calcification. Rates of calcification were measured for a coral, a crustose coralline alga, an echinoid, and a mollusk, using both approaches. For each group, the calcifying mass in incubations was varied and Ca^{2+}, A_T and all parameters known to influence A_T were measured.

MATERIALS AND METHODS

Mediterranean specimens of the scleractinian coral *Cladocora caespitosa*, the crustose coralline alga *Lithophyllum cabiochae*, the sea urchin *Paracentrotus lividus*, and the Mediterranean mussel *Mytilus galloprovincialis* were collected between March and May 2014 in the Bay of Villefranche (NW Mediterranean Sea, France; 43°40.73′N, 07°19.39′E). After collection, specimens were immediately transported to the Laboratoire d'Océanographie de Villefranche, cleaned of epiphytes and epibionts if necessary and kept under ~30 μmol photons m^{-2} s^{-1} in a temperature controlled room (16–19°C). Sampled mussels (ca. 5.5–6 cm shell length) were separated by carefully cutting their byssal threads and sea urchins were 5–8 cm in test diameter. The holding tanks received a continuous flow of external seawater pumped from a depth of 8 m (temperature: 16–18°C, salinity: ~38).

Within 2–4 d of collection, organisms were incubated in 5 l glass beakers previously cleaned with 10% HCl and rinsed with filtered seawater. Six beakers were filled with filtered (1 μm) seawater, gently bubbled with external air and mixed using a magnetic stirrer. Four of the 6 beakers were used to incubate the organisms, one was used to directly sample for initial conditions (t_0 hereafter), and another one was incubated without any organisms (the blank). In order to obtain a relatively wide range of calcification rates, incubations were conducted with various densities or masses of organisms (see Table 1). For corals and coralline algae, only the total incubated masses are reported in Table 1 due to the large variation in the sizes of the different pieces incubated. Mussels and sea urchins were incubated in darkness for 24 h, while coralline algae and corals were incubated both in the light (24 h under ~50 μmol photons m^{-2} s^{-1}) and in the dark (48 h). For each species, 2 series of incubations were conducted, leading to a sample size of 8 (i.e. 4 beakers with organisms × 2 series of incubations).

Before (in the t_0 beaker) and after incubation (in all beakers), samples were taken for the measurement of dissolved inorganic carbon (C_T), total alkalinity (A_T), calcium (Ca^{2+}), ammonium (NH_4^+), nitrate + nitrite ($NO_3^- + NO_2^-$) and phosphate (PO_4^{3-}). Samples for C_T (60 ml) were immediately poisoned with 100 μl of a saturated solution of mercuric chloride ($HgCl_2$) and analyzed within 2 d using an AIRICA (Automated Infra Red Inorganic Carbon Analyzer, Marianda) coupled to a LI-COR infrared gas analyser (LI-6262), on triplicate 1.2 ml subsamples at 22°C. The instrument was calibrated before every set of measurements using certified reference material (CRM) from A. Dickson (Scripps Institution of Oceanography, San Diego; Batch 132). Samples for A_T (300 ml) were filtered on GF/F membranes (47 mm), immediately poisoned with 100 μl of $HgCl_2$ and analyzed within 2 d. A_T was determined on triplicate 50 ml subsamples by potentiometric titration on a titrator (Titrando 888, Metrohm) coupled to a glass electrode (ecotrode plus, Metrohm) and a thermometer (pt1000). The pH electrode was calibrated daily on the total scale using Tris buffers of salinity 38 (salinity of the seawater used during the experiment). Measurements were carried out at 22°C and A_T was calculated as described by Dickson et al. (2007). During the experiment, standards provided by A. Dickson (Batch 132) were used to check precision (the SD within measurements of the same batch) and accuracy (deviation from the certified value provided by Dickson. Both precision and accuracy were 1.3 μmol kg^{-1} (n = 11). Samples for Ca^{2+} (100 ml) were filtered on GF/F membranes (47 mm) and kept in the refrigerator pending analysis (within 4 d). In the laboratory, Ca^{2+} was determined in

triplicate using the classic ethylene glycol tetraacetic acid (EGTA) potentiometric titration (Lebel & Poisson 1976). Approximately 10 g of seawater and 10 g of $HgCl_2$ solution (~1 mmol l^{-1}) were accurately weighed out; then ~10 g of a concentrated EGTA solution (~10 mmol l^{-1}, also by weighing) was added to completely complex Hg^{2+} and to complex nearly 95% of Ca^{2+}. After adding 10 ml of borate buffer (pH_{NBS} ~ 10) to increase the pH of the solution, the remaining Ca^{2+} was titrated with a diluted solution of EGTA (~2 mmol l^{-1}) using a titrator (Titrando 888) coupled to an amalgamated silver combined electrode (Ag Titrode, Metrohm). Following Cao & Dai (2011), the volume of EGTA necessary to titrate the remaining ~5% of Ca^{2+} was obtained by manually fitting a polynomial function to the first derivative of the titration curve using the R software (R Core Team 2014). The EGTA solution was calibrated prior to each measurement series using IAPSO (International Association for the Physical Sciences of the Oceans) standard seawater (Batch P147, Salinity: 35). NH_4^+ concentrations were measured using 2 different methods, depending on the expected concentrations. For the coral and coralline alga, they were measured in triplicate using a colorimetric technique (Holmes et al. 1999) and a laboratory fluorometer (Trilogy). For the mussel and sea urchin, for which higher concentrations were expected after incubation, unfiltered samples (60 ml) were immediately frozen at −20°C and measured on an autoanalyser (AA3 HR, Seal Analytical) at the Institute of Marine Science (Barcelona, Spain). Samples for the determination of $NO_3^- + NO_2^-$ and PO_4^{3-} (60 ml) were also immediately frozen and measured using an autoanalyser (AA3 HR, Seal Analytical) at the Laboratoire d'Océanographie de Villefranche.

Calcification rates (G_{A_T}) were estimated based on the alkalinity anomaly method, assuming that only net calcification affects A_T following the equation:

$$G_{A_T} = -\frac{\Delta A_T}{2} \quad (3)$$

Corrected calcification rates ($G^\star_{A_T}$) were estimated by considering that A_T is impacted by other processes. It was assumed that (1) the assimilation and remineralization of 1 mol of NH_4^+ (ΔNH_4^+) respectively lead to a decrease and an increase of 1 mol of A_T, (2) the assimilation and remineralization of 1 mol of $NO_3^- + NO_2^-$ ($\Delta(NO_3^- + NO_2^-)$) respectively lead to an increase and a decrease of 1 mol of A_T, and (3) the assimilation and remineralization of 1 mol of PO_4^{3-} (ΔPO_4^{3-}) respectively lead to an increase and a decrease of 1 mol of A_T. Therefore, $G^\star_{A_T}$ was estimated following the equation:

$$G^\star_{A_T} = \frac{\Delta NH_4^+ - \Delta A_T - \Delta NO_x - \Delta PO_4^{3-}}{2} \quad (4)$$

Finally, calcification rates (G_{Ca}) based on calcium variations (ΔCa^{2+}) were estimated following an inverse 1:1 relationship:

$$G_{Ca} = -\Delta Ca^{2+} \quad (5)$$

The carbonate chemistry was assessed using C_T and A_T and the R package seacarb (Lavigne et al. 2014). As most of the measured parameters have associated analytical errors (i.e. SDs from triplicate measurements), combined errors associated with the estimation of rates of change between t_0 and t_f (e.g. ΔA_T) as well as with the estimation of $G^\star_{A_T}$ were calculated as: $SE_{x-y} = \sqrt{SE_x^2 + SE_y^2}$. Model-II linear regressions (Sokal & Rohlf 1995) were used to compare net calcification rates obtained with the different methods. All regressions were performed using the R software (R Core Team 2014).

RESULTS

Environmental conditions during the incubations are shown in Table 1. For each species, 7 to 8 incubations were conducted, with a large range of incubated mass. All incubations were conducted under carbonate chemistry favorable to calcification, as shown by the lowest seawater saturation state with respect to aragonite measured during our experiment of 1.6. SDs associated with triplicate measurements of A_T and Ca^{2+} are presented in Fig. 1. SDs averaged 0.74 and 3.01 µmol kg^{-1} for A_T and Ca^{2+}, respectively (n = 76), corresponding to similar averaged CVs of ~0.03%.

The ranges of ΔA_T, ΔCa^{2+}, ΔNH_4^+ and ΔPO_4^{3-} observed during the incubations for each species are shown in Table 2. For all parameters, the changes observed for blank incubations were several orders of magnitude lower than those observed with organisms (data not shown). For all species, A_T and Ca^{2+} concentrations decreased during the incubations. The largest decreases in A_T and Ca^{2+} were observed for mussels (A_T: −582 and Ca^{2+}: −412 µmol kg^{-1} d^{-1}, corresponding to 267 and 580 g of mussels incubated). For the coral and the coralline alga, ΔNH_4^+, $\Delta(NO_3^- + NO_2^-)$ and ΔPO_4^{3-} were always more than 2 orders of magnitude lower than ΔA_T and ΔCa^{2+} (Table 2). Note that $\Delta(NO_3^- + NO_2^-)$ and ΔPO_4^{3-} could not be measured for the coral incubated in the dark. The processes impacting NH_4^+, $NO_3^- + NO_2^-$ and PO_4^{3-} contributed <2% of observed ΔA_T (Table 3).

Table 1. Environmental conditions during the incubations (8 incubations per species, except for corals in the light: n = 7). Ranges are presented for (min – max) of temperature (T), dissolved inorganic carbon (C_T), total alkalinity (A_T), pH on the total scale (pH_T), partial pressure of CO_2 (pCO_2), and saturation states with respect to aragonite ($\Omega_{Aragonite}$) and calcite ($\Omega_{Calcite}$). pH_T and pCO_2 were calculated at ambient temperature. Incubation times and the ranges of incubated mass (fresh weights) are also reported. Salinity was ~38

Species	Incubation time (h)	Incubated density (ind.)	Incubated mass (g)	T (°C)	C_T (μmol kg^{-1})	A_T (μmol kg^{-1})	pH_T	pCO_2 (μatm)	$\Omega_{Aragonite}$	$\Omega_{Calcite}$
C. caespitosa (coral)										
Light	24	–	126 – 816	16.8 – 19.0	2187 – 2275	2346 – 2524	8.0 – 8.1	317 – 563	2.4 – 3.3	3.8 – 5.1
Dark	48	–	96 – 677	16.0 – 17.0	2282 – 2325	2464 – 2533	7.8 – 8.0	572 – 723	2.1 – 2.5	3.2 – 3.8
L. cabiochae (coralline alga)										
Light	24	–	29 – 249	16.1 – 18.2	2041 – 2224	2273 – 2511	8.0 – 8.1	412 – 473	2.5 – 3.1	3.8 – 4.8
Dark	48	–	40 – 213	17.2 – 18.2	2247 – 2360	2453 – 2534	7.9 – 8.0	460 – 660	2.2 – 2.8	3.5 – 4.4
P. lividus (sea urchin)	24	1 – 5	39 – 190	16.0 – 16.2	2274 – 2343	2419 – 2558	7.8 – 8.0	551 – 892	1.8 – 2.5	2.8 – 3.8
M. galloprovincialis (mussel)	24	5 – 80	59 – 580	15.9 – 18.2	2151 – 2299	2299 – 2485	7.6 – 7.9	670 – 1379	1.6 – 2.1	2.4 – 3.3

The uncorrected or corrected alkalinity anomaly (G_{A_T} or $G^*_{A_T}$) and calcium anomaly (G_{Ca}) techniques provided similar calcification rates and no statistical differences between rates were found for the coral incubated in the light and in the dark (Table 4, Fig. 2). Similar results were obtained for the crustose coralline alga incubated in the dark with a 1:1 relationship between the estimates. However, for the coralline alga incubated in the light, G_{A_T} and $G^*_{A_T}$ were significantly higher than G_{Ca} (Table 4, Fig. 2).

In contrast to the coral and coralline alga, the mussel and the sea urchin were significant sources of ammonium during the incubations. ΔNH_4^+ was of the same order of magnitude as ΔA_T and ΔCa^{2+} (Table 2). In both species, NH_4^+ release through excretion was the second most important process affecting A_T, representing 36.2 ± 6.3 and $17.1 \pm 2.6\%$ of ΔA_T for the

sea urchin and the mussel, respectively (Table 3). Variations in $NO_3^- + NO_2^-$ and PO_4^{3-} contributed 0.1 ± 0.1 to $1.8 \pm 0.6\%$ of ΔA_T. For the sea urchin, calcification rates as estimated by the alkalinity anomaly technique without any correction provided slightly different values than G_{Ca} (Fig. 3, Table 4). After correction for nutrient variations, $G^*_{A_T}$ values were significantly higher than G_{Ca}. For the mussel, estimates based on the alkalinity anomaly technique provided rates that were significantly lower than those estimated by the calcium anomaly technique (Fig. 3, Table 4). For the lowest range of densities and calcification rates (<200 μmol kg^{-1} d^{-1}), the correction applied to the alkalinity anomaly technique led to rates that were similar to the ones based on calcium. At higher densities and calcification rates (>200 μmol kg^{-1} d^{-1}), although the corrections tended to move calcification rates closer to values estimated using the calcium anomaly method, calcification rates were still significantly lower than rates based on calcium (Fig. 3).

DISCUSSION

Significant changes in A_T and Ca^{2+} were observed during incubations of the 4 species considered and at all abundances. For the coral, results are consistent with previous studies that reported very small impacts of nutrient consumption and release on A_T and no significant differences between rates of calcification estimated using the alkalinity and calcium anomaly techniques (Chisholm & Gattuso 1991, Murillo et al. 2014). Note that Murillo et al. (2014) found that calcification rates estimated by the alkalinity anomaly technique were significantly lower
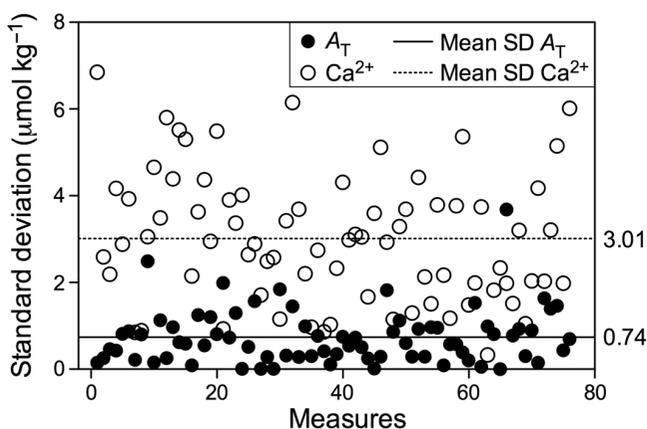

Fig. 1. SDs of triplicate measurements of total alkalinity (A_T) and calcium (Ca^{2+}) for all measurements (n = 76). Horizontal lines are mean SDs (n = 76) for A_T (—) and Ca^{2+} (---)

Table 2. Amplitude of changes (µmol kg^{-1} t^{-1}; t = 24 h in the light and 48 h in the dark) in total alkalinity (ΔA_T), calcium (ΔCa^{2+}), ammonium (ΔNH_4^+), nitrate+nitrite ($\Delta(NO_3^-+NO_2^-)$) and phosphate (ΔPO_4^{3-}) observed during the incubations (8 incubations per species, except for the coral in the light: n = 7). NA: not available

| Species | | ΔA_T | | ΔCa^{2+} | | ΔNH_4^+ | | $\Delta(NO_3^-+NO_2^-)$ | | ΔPO_4^{3-} | |
		Max	Min	Max	Min	Max$_{\Delta<0}$ Min$_{\Delta>0}$	Max	Max$_{\Delta<0}$ Min$_{\Delta>0}$	Max	Max$_{\Delta<0}$ Min$_{\Delta>0}$	Max
C. caespitosa (coral)	Light	−415	−59	−189	−38	−0.1	2.7	−4.0	4.0	−0.1	0.0
	Dark	−172	−28	−68	−9	0.5	3.0	NA	NA	NA	NA
L. cabiochae (coralline alga)	Light	−577	−100	−218	−23	−0.4	0.7	−1.5	0.2	−0.2	0.1
	Dark	−187	−69	−90	−16	−0.6	2.7	−0.7	1.8	−0.1	0.4
P. lividus (sea urchin)		−293	−3	−151	−13	80	159	−0.8	0.9	−0.8	1.4
M. galloprovincialis (mussel)		−582	−194	−412	−111	33	182	0.4	12	5.4	22

Table 3. Contribution (% ± SE) of the considered processes to observed variation in total alkalinity. ΔNH_4^+, $\Delta(NO_3^-+NO_2^-)$, ΔPO_4^{3-}: assimilation or remineralization of ammonium, nitrate+nitrite and phosphate, respectively. G: net calcification. NA: not available

Species		ΔNH_4^+	$\Delta(NO_3^-+NO_2^-)$	ΔPO_4^{3-}	G
C. caespitosa (coral)	Dark	1.9 ± 2.1	NA	NA	98.1 ± 2.1
	Light	0.5 ± 0.7	1.2 ± 1.0	0.0 ± 0.0	98.3 ± 1.6
L. cabiochae (coralline alga)	Dark	0.6 ± 0.7	0.5 ± 0.4	0.1 ± 0.1	98.8 ± 1.1
	Light	0.2 ± 0.1	0.3 ± 0.3	0.0 ± 0.0	99.5 ± 0.7
P. lividus (sea urchin)	Dark	36.2 ± 6.3	0.1 ± 0.1	0.2 ± 0.1	63.6 ± 6.3
M. galloprovincialis (mussel)	Dark	17.1 ± 2.6	0.9 ± 1.6	1.8 ± 0.6	80.2 ± 2.6

Table 4. Outputs of the model-II regressions (n = 8 incubations per species, except for corals in the light: n = 7) between net calcification rates derived from the alkalinity anomaly technique (uncorrected: G_{A_T} or corrected: $G^*_{A_T}$) and the calcium anomaly technique (G_{Ca}): G_{A_T} or $G^*_{A_T}$ = Slope ± SE × G_{Ca} + Y-intercept ± SE. p-values of Student's t-tests for the slope and the Y-intercept are presented. **Bold** values denote significant differences at $\alpha < 0.05$

			n	Slope	Y-intercept	Slope ≠ 0 p-value	Slope ≠ 1 p-value	Y-intercept ≠ 0 p-value
C. caespitosa (coral)	Dark	G_{A_T}	8	1.02 ± 0.08	2.8 ± 3.3	**< 0.001**	0.82	0.42
		$G^*_{A_T}$	8	1.02 ± 0.08	3.4 ± 3.3	**< 0.001**	0.85	0.34
	Light	G_{A_T}	7	1.15 ± 0.07	−19.1 ± 7.8	**< 0.001**	0.08	0.05
		$G^*_{A_T}$	7	1.15 ± 0.07	−19.4 ± 8.1	**< 0.001**	0.09	0.05
L. cabiochae (coralline alga)	Dark	G_{A_T}	8	1.15 ± 0.08	−12.1 ± 5.9	**< 0.001**	0.11	0.09
		$G^*_{A_T}$	8	1.16 ± 0.07	−12.9 ± 5.2	**< 0.001**	0.05	0.05
	Light	G_{A_T}	8	1.18 ± 0.08	31.2 ± 10.7	**< 0.001**	0.06	**0.03**
		$G^*_{A_T}$	8	1.18 ± 0.08	31.4 ± 10.7	**< 0.001**	0.06	**0.03**
P. lividus (sea urchin)		G_{A_T}	8	1.08 ± 0.07	−16.2 ± 5.1	**< 0.001**	0.29	**0.02**
		$G^*_{A_T}$	8	1.40 ± 0.12	21.9 ± 8.7	**< 0.001**	**0.01**	0.05
M. galloprovincialis (mussel)		G_{A_T}	8	0.60 ± 0.04	32.4 ± 12.3	**< 0.001**	**< 0.001**	**0.04**
		$G^*_{A_T}$	8	0.79 ± 0.05	26.1 ± 14.3	**< 0.001**	**0.005**	0.12

than calcium-based estimates when the whole reef community, including sediment, was considered. This discrepancy was attributed to the release of A_T from the sediment as a consequence of anaerobic mineralization processes, such as net sulphate reduc-

tion and denitrification (Hu & Cai 2011). No correction for nutrient release was necessary in our study for isolated colonies of *Cladocora caespitosa* and errors associated with A_T measurements were relatively low (Fig. 2). Consequently, calcification rates
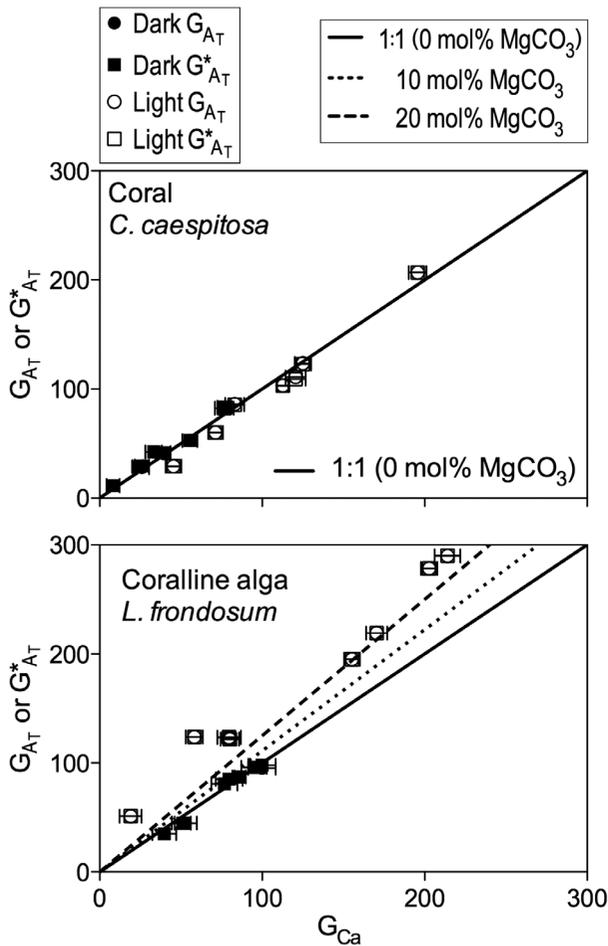
Fig. 2. Calcification rates (mean ± SE; µmol kg^{-1} t^{-1}; t = 48 h in the dark and 24 h in the light) estimated based on the alkalinity anomaly technique (uncorrected: G_{AT} or corrected: G^*_{AT}) as a function of calcification rates estimated based on the calcium anomaly technique (G_{Ca}) for the scleractinian coral *Cladocora caespitosa* (upper panel) and the crustose coralline alga *Lithophyllum cabiochae* (lower panel). G_{AT} and G^*_{AT} are not different from each other and are superimposed. SEs for G_{AT} and G^*_{AT} are too small to be visible. Lines represent the theoretical relationship between G_{AT} or G^*_{AT} and G_{Ca} for different contents of magnesiun in the skeleton (%MgCO$_3$)

Fig. 3. Calcification rates (mean ± SE; µmol kg^{-1} t^{-1}; t = 24 h) estimated based on the alkalinity anomaly technique (uncorrected: G_{AT} or corrected: G^*_{AT}) as a function of calcification rates estimated based on the calcium anomaly technique (G_{Ca}) for the sea urchin *Paracentrotus lividus* (upper panel) and the mussel *Mytilus galloprovincialis* (lower panel). G_{AT} and G^*_{AT} are not different from each other and are superimposed. SEs for G_{AT} and G^*_{AT} are too small to be visible. Lines represent the theoretical relationship between G_{AT} or G^*_{AT} and G_{Ca} for different contents of magnesium in the skeleton or shells (%MgCO$_3$)

could be estimated with very good precision for this species.

The average precision (SD) of A_T was ~0.7 µmol kg^{-1}, which is below the precision specified by Dickson et al. (2007) for open-cell titrations (~1 µmol kg^{-1}). The precision of Ca^{2+} measurements was significantly less than for A_T (Fig. 1; ~3 µmol kg^{-1}). Since there is a theoretical 1:1 relationship between calcification and ΔCa^{2+} for organisms precipitating calcite and aragonite, the calcium anomaly technique is less efficient in measuring low rates of calcification (low calcifying mass and/or small incubation times) and in
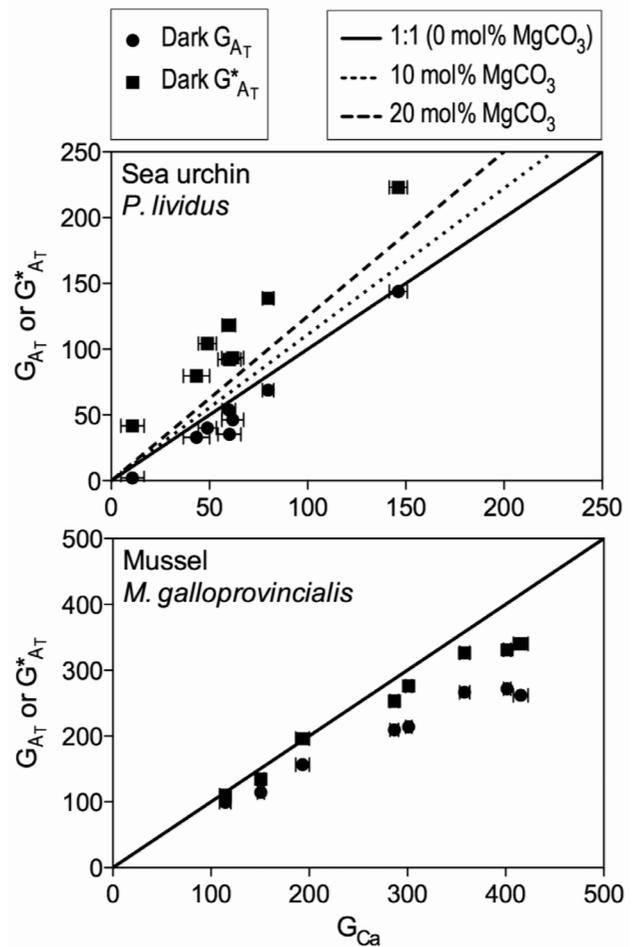
capturing small differences in calcification as a consequence of varying environmental conditions, such as ocean acidification. It must be stressed that the precision reached in our study is among the highest reported for Ca^{2+} in seawater, using the same protocol. Indeed, Cao & Dai (2011) reported averaged SDs of ~6 µmol kg^{-1}, while Murillo et al. (2014), using a similar technique based on titrations with EGTA but using a Ca^{2+}-selective electrode, reported measurement errors of ~20 µmol kg^{-1}. Recently, Liu et al. (2014) reviewed available techniques for measuring Ca^{2+} in seawater and the precision reached in our

study (~0.03% of relative SD) was better than all studies using a similar protocol (EGTA titration; >0.1%) and lower than that based on isotope dilution and inductively coupled plasma mass spectrometry (ID-ICP; 0.1%) or thermal ionization mass spectrometry (ID-TIMS; 0.05%). The main advantage of mass spectrometry techniques is the very low volume of sample necessary for the measurements (~10 µl for ID-ICP; Liu et al. 2014). Nevertheless, following these considerations, the use of the alkalinity anomaly technique for measuring calcification rates of isolated corals appears to be a better choice, as this method allows the detection of rates of calcification or difference in calcification rates above ~0.5 µmol kg^{-1} incubation^{-1}, assuming robust analytical protocols.

As with the coral, the crustose coralline alga *Lithophyllum cabiochae* showed variations in nutrient concentrations resulting from nutrient acquisition/ release that were relatively low compared to ΔA_T; thus, no correction of the alkalinity anomaly technique was necessary. Calcification rates estimated by the 2 methods were significantly correlated. While rates were not significantly different in the dark, the alkalinity anomaly technique provided rates in the light that were significantly lower than those based on calcium anomaly. This suggests that this coralline alga produces magnesian calcite during the day, but not at night. It is well established that calcareous algae precipitate calcite with a significant proportion of Mg^{2+} (Diaz-Pulido et al. 2014), but to the best of our knowledge, no studies have reported such difference between calcification under light and dark conditions. The lack of net Mg^{2+} incorporation at night could be due to chemical conditions being less favorable for this mineral that is more soluble than 'pure' calcite. The release of CO_2 through respiration and the lack of CO_2 consumption through photosynthesis could lead to less favorable conditions in the immediate surrounding (diffusion boundary layer) of the coralline alga at night. Several studies have recently shown an impact of ocean acidification with or without warming on the $MgCO_3/CaCO_3$ ratio of coralline algae (e.g. Ries 2011, Egilsdottir et al. 2013, Diaz-Pulido et al. 2014). Our data suggest that the response to these drivers could be modulated by irradiance, and that future experiments should cover the range of irradiance to which the coralline algae are exposed in the field. Although the slopes of the regressions between G_{A_T} or $G^\star_{A_T}$ and G_{Ca} were not significantly different from 1, one can see in Fig. 3 that points are close to the theoretical line for calcite containing 20 mol% of $MgCO_3$. This is slightly above the $MgCO_3$ contents of 15 mol% reported for the

same species sampled at the same location by S. Martin et al. (unpubl.). It must be stressed that the irradiance levels during our incubations were significantly higher than the levels considered by S. Martin et al. (unpubl.) (9–35 µmol photons m^{-2} s^{-1}) and could explain the higher proportion of $MgCO_3$ precipitation in our study. However, the lowest rates of calcification appeared to depart from this theoretical line, with Y-intercepts that were significantly different from 0 (Table 4). The reasons of these discrepancies remain unclear. As for the coral, overall, the alkalinity anomaly technique appears precise and accurate enough to estimate calcification rates for this species, and is especially well suited to detecting changes in calcification rates as a consequence of environmental change. In contrast, in the light, measuring Ca^{2+} alone without considering Mg^{2+} incorporation does not allow accurate estimation of net calcification rates. [$Ca^{2+} + Mg^{2+} + $ strontium] can be determined in seawater by a potentiometric titration similar to the one used for Ca^{2+} in the present paper. The Mg^{2+} concentration can be obtained by difference, assuming that the strontium concentration is negligible (~0.2% of Mg^{2+} concentration). However, estimating changes in Mg^{2+} concentrations is even more challenging than estimating changes in Ca^{2+} because it is based on 2 titrations, each with associated analytical errors. Furthermore, the background concentration of Mg^{2+} is very large, with a Mg^{2+}/Ca^{2+} ratio of ~5.2, making detection of small changes very difficult.

In contrast to the coral and the coralline alga, changes in the concentrations of NH_4^+, $NO_3^-+NO_2^-$ and PO_4^{3-} in the sea urchin and the mussel were important relative to net calcification and were required to correct changes in A_T. Among processes involved, changes in NH_4^+ concentrations were responsible for 17 to 36% of changes in A_T. NH_4^+ originates from the ammonification of organic waste (feces, excess food, dead animals) by heterotrophic bacteria that use these wastes as a source of nutrients. It is also generated by animal excretion and results from deamination and transamination during food digestion. Echinoids excrete urea, which is readily decomposed by ureolytic bacteria into ammonia (Basuyaux & Mathieu 1999). In contrast, mussels are known to excrete mostly nitrogen in the form of NH_4^+ (40 to 100%), the remaining nitrogen being excreted in an organic form (Bayne & Scullard 1977). There are very few reports on the influence of NH_4^+ excretion by echinoids and mollusks on A_T. Previous experiments that focused on the effect of ocean acidification on bivalve calcification assumed that NH_4^+

excretion is negligible (Gazeau et al. 2007, Mingliang et al. 2011, Waldbusser et al. 2011). Recently, however, Gazeau et al. (2014) reported NH_4^+ excretion rates being responsible for 60% (on average) of ΔA_T in the Mediterranean mussel *Mytilus galloprovincialis* kept in the laboratory for ~1 yr. This contribution is much higher than that reported in the present study, suggesting that correction of ΔA_T could vary significantly depending on the physiological state of the organism. In contrast to bivalves, the alkalinity anomaly technique has not been used to estimate echinoid calcification, to the best of our knowledge. So far, the buoyant weight (Ries et al. 2009) and the calcium content of calcified structures (Wood et al. 2008) have been the preferred techniques, although these are destructive and/or are applicable only over relatively long cultivation periods.

Sea urchins are also known to produce relatively large amounts of magnesian calcite (Weber 1969), explaining why calcification rates estimated based on Ca^{2+} anomaly were significantly lower than those obtained from changes in A_T corrected for changes in nutrients. However, the correction of the alkalinity anomaly technique led to estimates of net calcification rates much above the theoretical line considering 20 mol% of $MgCO_3$, which seems to be a high estimate for this species as a ratio of 11.8 mol% has been reported for *Paracentrotus lividus* in the Mediterranean Sea (Hermans et al. 2010). As such, our study does not allow us to test the validity of the alkalinity technique for this species and further comparative studies should consider measuring Mg^{2+} incorporation rates.

In the mussel, in addition to changes in NH_4^+ concentrations, changes in both $NO_3^- + NO_2^-$ and PO_4^{3-} were responsible for ~1 and 2% of ΔA_T, respectively. Excretion might have released PO_4^{3-}, while the increase in $NO_3^- + NO_2^-$ was most likely due to the nitrification of a small proportion of the excreted NH_4^+. Indeed, mollusks are known to shelter nitrifying bacteria in their tissues or on the shells (Welsh & Castadelli 2004). Although the contribution of this process was rather small in our study, it could be much larger in other species or in different environmental settings. It is recommended that correcting for changes in $NO_3^- + NO_2^-$ be considered when using the alkalinity anomaly technique with mollusks.

Mussels precipitate low amounts of both magnesian calcite and aragonite (Dodd 1965); therefore, the calcium anomaly technique should provide accurate rates of calcification. The corrected alkalinity anomaly technique provided results very similar to those based on Ca^{2+} in the lowest range of calcification

rates (0–200 µmol kg^{-1} d^{-1}); however, at the highest mussel densities and calcification rates, calcification rates estimated by the alkalinity anomaly method were lower than those derived from ΔCa^{2+}, even after correcting for changes in nutrient concentrations. This discrepancy could be due to 2 non-exclusive hypotheses. First, final ammonium concentrations corresponding to the highest calcification rates were >120 µmol kg^{-1}, which is well above the range of concentrations usually measured using the colorimetric method employed in our study. It cannot be ruled out that these concentrations have been significantly underestimated as a consequence of storage and/or necessary multiple dilutions. Second, it is possible that another process affecting A_T was not taken into account. As mentioned previously, mussels excrete a significant proportion of N as organic wastes. Dissolved organic compounds are believed to substantially contribute to seawater total alkalinity (Kim & Lee 2009) and have been shown to be a significant buffering component in organic-rich river systems (Hunt et al. 2011). Release of dissolved organic carbon (DOC) was measured during our experiment (data not shown) but cannot account for this missing process. DOC release by *M. galloprovincialis* represented <3% of ΔA_T, assuming that 100% of this DOC is charged and contributes to A_T; therefore, it is unlikely that ΔDOC was responsible for significant variations in A_T during our experiments. Except for some species that contain a few percent of $MgCO_3$ (e.g. Ries 2011), marine mollusks precipitate low amounts of magnesian calcite and/or aragonite. If an analytical technique as precise as the one presented in this study is used, the calcium anomaly technique appears to be a valid method to estimate calcification rates for these species. Considering that A_T is significantly affected by nutrients and especially NH_4^+ excretion, it is strongly recommended to use the alkalinity anomaly technique for mollusks only after appropriate corrections. Furthermore, low incubation densities should be preferred in order to maintain ammonium concentrations at accurately measurable levels. Since changes in A_T as low as 0.5 µmol kg^{-1} incubation^{-1} can be accurately measured, there is no real need to reach calcification rates >200–500 µmol kg^{-1} incubation^{-1} as we did in this study.

In the framework of perturbation studies (e.g. ocean acidification research), it would be tempting to consider that the alkalinity anomaly technique without correction is still useful when comparing rates among treatments (different pH levels) in a given study, despite not providing accurate absolute rates. How-

ever, as both net calcification and excretion rates are potentially impacted by ocean acidification, this should be considered with caution. For instance, Thomsen & Melzner (2010) reported that shell growth of *Mytilus edulis* from the Baltic Sea decreased linearly with increasing pCO_2, while NH_4^+ excretion increased linearly. The same observations were made by Fernandez-Reiriz et al. (2012) and Range et al. (2012) for *M. galloprovincialis* from the European Atlantic coast. In contrast, Range et al. (2014) showed that while shell thickness and integrity of *M. galloprovincialis* from the Adriatic Sea decreased at low pH, suggesting lower calcification rates, excretion did not appear to be as sensitive. Since there is no clear consensus on the effect of ocean acidification on N excretion of shelled mollusks (Gazeau et al. 2013) and in order to avoid any misinterpretations, we strongly recommend systematically measuring changes in NH_4^+ while estimating calcification rates based on A_T variations.

In conclusion, our experiment confirmed previous studies showing that the alkalinity and calcium anomaly techniques provide similar rates of calcification in zooxanthellate corals. Since measurements of A_T have low analytical error and changes in A_T are twice larger than changes in Ca^{2+} (and hence easier to detect), the alkalinity anomaly technique is well suited for measuring low calcification rates and should therefore be preferred. It also appears well suited for crustose coralline algae, especially when maintained in the light. In contrast, for mussels and sea urchins, the alkalinity anomaly technique requires corrections to take into account the effect of nutrient release (especially ammonium) on A_T. This technique has a relative uncertainty in mussels at the highest rates of calcification and N excretion, even after correction. Hence, it is recommended that the alkalinity anomaly technique be used for mollusks only following correction for N excretion and at low incubation densities. At higher densities, the less precise calcium anomaly technique appears to be the best alternative since most mollusks precipitate low amounts of magnesian calcite and/or aragonite. For sea urchins, the validity of the alkalinity anomaly technique could not be fully tested, although it appears to be more valid than the calcium anomaly technique; as these organisms produce high amounts of magnesian calcite, both changes in Ca^{2+} and Mg^{2+} need to be measured. Moreover, changes in Mg^{2+} remain difficult to precisely estimate in seawater due to high background concentrations and low incorporation rates.

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