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

F. Gazeau1,2,*, L. Urbini1,2,3, T. E. Cox1,2, S. Alliouane1,2, J.-P. Gattuso1,2

1Sorbonne Universités, UPMC Univ Paris 06, UMR 7093, Laboratoire d’Océanographie de Villefranche (LOV), Observatoire Océanologique, Villefranche-sur-mer 06230, France
2CNRS, UMR 7093, Laboratoire d’Océanographie de Villefranche (LOV), Observatoire Océanologique, Villefranche-sur-mer 06230, France
3Università degli Studi di Genova (DiSTAV), Corso Europa 26, Genova 16132, Italy

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 methods are not robustly applicable to all calcifiers without careful consideration of organism physiology and mineralogy.

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 (CaCO₃). It plays a major role in the global carbon cycle over different time scales (Milliman 1993) and is the key mechanism by which CO₂ is returned to the atmosphere (Berner et
The capacity of marine organisms to produce CaCO₃ 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 CO₂ 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 ([CO₃²⁻]) and increases in CO₂ and bicarbonate concentrations ([HCO₃⁻]) 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 factors (Hansson 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 (⁴⁵Ca, Goreau & Bowen 1955; ¹⁴C, Paasche 1963) in CaCO₃ have also been used. These techniques involve incubations in seawater with or without ⁴⁵Ca or ¹⁴C. The organism must subsequently be sacrificed and the skeletal components dissolved in acid to measure C, Ca²⁺, ¹⁴C or ⁴⁵Ca. 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):

\[ A_T = [HCO_3^-] + 2[CO_3^{2-}] + [Br(OH)_2^-] + [OH^-] + [HPO_4^{2-}] + 2[PO_4^{3-}] + [H_2SiO_4^-] + [NH_4^+] + [HS^-] + [HSO_4^-] + [H^+] - [HF] - [H_3PO_4^-] + [minor acids – minor bases] \]  

(1)

As can be seen in Eq. (1), A_T is highly influenced by [HCO₃⁻] and [CO₃²⁻] together with a multitude of other minor compounds. Calcification consumes carbonate or bicarbonate, following the reversible reaction:

\[ Ca^{2+} + 2HCO_3^- \rightleftharpoons CaCO_3 + CO_2 + H_2O \]  

(2)

Eq. (2) shows that calcification consumes 2 moles of HCO₃⁻, therefore decreasing A_T by 2 moles per mole of CaCO₃ 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; coraline 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 (Ca²⁺) 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 Ca²⁺ 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). Sample 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$^{-}$), total alkalinity ($A_T$), calcium (Ca$^{2+}$), ammonium (NH$_4^+$), nitrate + nitrite (NO$_3^−$+NO$_2^−$) and phosphate (PO$_4^{3−}$). Samples for C$^{-}$ (60 ml) were immediately poisoned with 100 μl of a saturated solution of mercuric chloride (HgCl$_2$) and analyzed within 2 d using an AIRA C 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 (Titrand 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 HgCl₂ 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 Hg²⁺ and to complex nearly 95% of Ca²⁺. After adding 10 ml of borate buffer (pH 8.2) to increase the pH of the solution, the remaining Ca²⁺ 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 Ca²⁺ 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). NH₄⁺ 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 Laboratoire d’Océanographie de Villefranche. Samples for the determination of NO₃⁻+NO₂⁻ and PO₄³⁻ (60 ml) were also immediately frozen and measured using an autoanalyser (AA3 HR, Seal Analytical) at the Laboratoire d’Océanographie de Villefranche.

Calibration rates (GₐT) were estimated based on the alkalinity anomaly method, assuming that only net calcification affects Aₜ following the equation:

\[ G_{A_T} = -\frac{\Delta A_T}{2} \]  

(3)

Corrected calibration rates (Gₐ*ₜ) were estimated by considering that Aₜ is impacted by other processes. It was assumed that (1) the assimilation and remineralization of 1 mol of NH₄⁺ (ΔNH₄⁺) respectively lead to a decrease and an increase of 1 mol of Aₜ, (2) the assimilation and remineralization of 1 mol of NO₃⁻+NO₂⁻ (Δ(NO₃⁻+NO₂⁻)) respectively lead to an increase and a decrease of 1 mol of Aₜ, and (3) the assimilation and remineralization of 1 mol of PO₄³⁻ (ΔPO₄³⁻) respectively lead to an increase and a decrease of 1 mol of Aₜ. Therefore, Gₐ*ₜ was estimated following the equation:

\[ G_{A_T} = \frac{\Delta NH_4^+ - \Delta A_T - \Delta NO_3^- - \Delta PO_4^{3-}}{2} \]  

(4)

Finally, calcification rates (GₐCa) based on calcium variations (ΔCa²⁺) were estimated following an inverse 1:1 relationship:

\[ G_{Ca} = -\Delta Ca^{2+} \]  

(5)

The carbonate chemistry was assessed using Cₜ and Aₜ 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 t₀ and tₜ (e.g. ΔAₜ) as well as with the estimation of GₐCa were calculated as: \( SE_{x,y} = \sqrt{SE_{x}^2 + SE_{y}^2} \). Model-II linear regressions (Sokal & Rohl 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 Aₜ and Ca²⁺ are presented in Fig. 1. SDs averaged 0.74 and 3.01 μmol kg⁻¹ for Aₜ and Ca²⁺, respectively (n = 76), corresponding to similar averaged CVs of ~0.03%.

The ranges of ΔAₜ, ΔCa²⁺, ΔNH₄⁺ and ΔPO₄³⁻ 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, Aₜ and Ca²⁺ concentrations decreased during the incubations. The largest decreases in Aₜ and Ca²⁺ were observed for mussels (Aₜ: ~582 and Ca²⁺: ~412 μmol kg⁻¹ d⁻¹, corresponding to 267 and 580 g of mussels incubated). For the coral and the coralline alga, ΔNH₄⁺, Δ(NO₃⁻+NO₂⁻) and ΔPO₄³⁻ were always more than 2 orders of magnitude lower than ΔAₜ and ΔCa²⁺ (Table 2). Note that Δ(NO₃⁻+NO₂⁻) and ΔPO₄³⁻ could not be measured for the coral incubated in the dark. The processes impacting NH₄⁺, NO₃⁻+NO₂⁻ and PO₄³⁻ contributed <2% of observed ΔAₜ (Table 3).
The uncorrected or corrected alkalinity anomaly (GAT or G*AT) and calcium anomaly (GCa) 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, GAT and G*AT were significantly higher than GCa (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. ΔNH₄⁺ was of the same order of magnitude as ΔAT and ΔCa²⁺ (Table 2). In both species, NH₄⁺ release through excretion was the second most important process affecting AT, representing 36.2 ± 6.3 and 17.1 ± 2.6% of ΔAT for the sea urchin and the mussel, respectively (Table 3). Variations in NO₃⁻+NO₂⁻ and PO₄³⁻ contributed 0.1 ± 0.1 to 1.8 ± 0.6% of ΔAT. For the sea urchin, calcification rates as estimated by the alkalinity anomaly technique without any correction provided slightly different values than GCa (Fig. 3, Table 4). After correction for nutrient variations, G*AT values were significantly higher than GCa. 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 AT and Ca²⁺ 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 AT 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...
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 reduction 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

Table 2. Amplitude of changes (μmol kg$^{-1}$; $t = 24$ h in the light and 48 h in the dark) in total alkalinity ($\Delta A_T$), calcium ($\Delta Ca^{2+}$), ammonium ($\Delta NH_4^+$), nitrate+nitrite ($\Delta(NO_3^-+NO_2^-)$) and phosphate ($\Delta PO_4^{3-}$) observed during the incubations (8 incubations per species, except for the coral in the light: $n = 7$). NA: not available

<table>
<thead>
<tr>
<th>Species</th>
<th>$\Delta A_T$</th>
<th>$\Delta Ca^{2+}$</th>
<th>$\Delta NH_4^+$</th>
<th>$\Delta(NO_3^-+NO_2^-)$</th>
<th>$\Delta PO_4^{3-}$</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max</td>
<td>Min</td>
<td>Max</td>
<td>Min</td>
<td>Max</td>
<td>Min</td>
</tr>
<tr>
<td>C. caespitosa (coral)</td>
<td>Light</td>
<td>−415</td>
<td>−59</td>
<td>−189</td>
<td>−38</td>
<td>−0.1</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>−172</td>
<td>−28</td>
<td>−68</td>
<td>−9</td>
<td>0.5</td>
</tr>
<tr>
<td>L. cabiochae (coralline alga)</td>
<td>Light</td>
<td>−577</td>
<td>−100</td>
<td>−218</td>
<td>−23</td>
<td>−0.4</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>−187</td>
<td>−69</td>
<td>−90</td>
<td>−16</td>
<td>−0.6</td>
</tr>
<tr>
<td>P. lividus (sea urchin)</td>
<td>−293</td>
<td>−3</td>
<td>−151</td>
<td>−13</td>
<td>80</td>
<td>159</td>
</tr>
<tr>
<td>M. galloprovincialis (mussel)</td>
<td>−582</td>
<td>−194</td>
<td>−412</td>
<td>−111</td>
<td>33</td>
<td>182</td>
</tr>
</tbody>
</table>

Table 3. Contribution (% ± SE) of the considered processes to observed variation in total alkalinity. $\Delta NH_4^+$, $\Delta(NO_3^-+NO_2^-)$, $\Delta PO_4^{3-}$: assimilation or remineralization of ammonium, nitrate+nitrite and phosphate, respectively. G: net calcification. NA: not available

<table>
<thead>
<tr>
<th>Species</th>
<th>$\Delta NH_4^+$</th>
<th>$\Delta(NO_3^-+NO_2^-)$</th>
<th>$\Delta PO_4^{3-}$</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. caespitosa (coral)</td>
<td>Dark</td>
<td>1.9 ± 2.1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>0.5 ± 0.7</td>
<td>1.2 ± 1.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>L. cabiochae (coralline alga)</td>
<td>Dark</td>
<td>0.6 ± 0.7</td>
<td>0.5 ± 0.4</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.3</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>P. lividus (sea urchin)</td>
<td>Dark</td>
<td>36.2 ± 6.3</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>M. galloprovincialis (mussel)</td>
<td>Dark</td>
<td>17.1 ± 2.6</td>
<td>0.9 ± 1.6</td>
<td>1.8 ± 0.6</td>
</tr>
</tbody>
</table>

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: $G_A$ or corrected: $G_A'$) and the calcium anomaly technique ($G_{Ca}$): $G_A$ or $G_A' = $ Slope ± SE $\times 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 $\alpha < 0.05$

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Slope</th>
<th>Y-intercept</th>
<th>Slope ≠ 0</th>
<th>Slope ≠ 1</th>
<th>Y-intercept ≠ 0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p-value</td>
<td>p-value</td>
<td>p-value</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>C. caespitosa (coral)</td>
<td>Dark</td>
<td>$G_A$</td>
<td>8</td>
<td>1.02 ± 0.08</td>
<td>2.8 ± 3.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$G_A'$</td>
<td>8</td>
<td>1.02 ± 0.08</td>
<td>3.4 ± 3.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>$G_A$</td>
<td>7</td>
<td>1.15 ± 0.07</td>
<td>−19.1 ± 7.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$G_A'$</td>
<td>7</td>
<td>1.15 ± 0.07</td>
<td>−19.4 ± 8.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>L. cabiochae (coralline alga)</td>
<td>Dark</td>
<td>$G_A$</td>
<td>8</td>
<td>1.15 ± 0.08</td>
<td>−12.1 ± 5.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$G_A'$</td>
<td>8</td>
<td>1.16 ± 0.07</td>
<td>−12.9 ± 5.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>$G_A$</td>
<td>8</td>
<td>1.18 ± 0.08</td>
<td>31.2 ± 10.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$G_A'$</td>
<td>8</td>
<td>1.18 ± 0.08</td>
<td>31.4 ± 10.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P. lividus (sea urchin)</td>
<td>Dark</td>
<td>$G_A$</td>
<td>8</td>
<td>1.08 ± 0.07</td>
<td>−16.2 ± 5.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$G_A'$</td>
<td>8</td>
<td>1.40 ± 0.12</td>
<td>21.9 ± 8.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M. galloprovincialis (mussel)</td>
<td>Dark</td>
<td>$G_A$</td>
<td>8</td>
<td>0.60 ± 0.04</td>
<td>32.4 ± 12.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$G_A'$</td>
<td>8</td>
<td>0.79 ± 0.05</td>
<td>26.1 ± 14.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
could be estimated with very good precision for this species.

The average precision (SD) of $A_T$ was $\sim 0.7$ 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
the MgCO$_3$ contents of 15 mol% reported for the release that were relatively low compared to concentrations resulting from nutrient acquisition/phyllum cabiochae showed variations in nutrient of Mg$^{2+}$ (Diaz-Pulido et al. 2014), but to the best of our knowledge, no studies have reported such 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$_{AT}$ or G$_{AT}$ and C$_{Ca}$ 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 NH₄⁺ excretion rates being responsible for 60% (on average) of ΔAₜ 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 ΔAₜ 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 Ca²⁺ anomaly were significantly lower than those obtained from changes in Aₜ 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 MgCO₃, 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 Mg²⁺ incorporation rates.

In the mussel, in addition to changes in NH₄⁺ concentrations, changes in both NO₃⁻+NO₂⁻ and PO₄³⁻ were responsible for ~1 and 2% of ΔAₜ, respectively. Excretion might have released PO₄³⁻, while the increase in NO₃⁻+NO₂⁻ was most likely due to the nitrification of a small proportion of the excreted NH₄⁺. 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 NO₃⁻+NO₂⁻ 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 Ca²⁺ 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 ΔCa²⁺, 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 Aₜ 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 ΔAₜ, assuming that 100% of this DOC is charged and contributes to Aₜ; therefore, it is unlikely that ΔDOC was responsible for significant variations in Aₜ during our experiments.

Except for some species that contain a few percent of MgCO₃ (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 Aₜ is significantly affected by nutrients and especially NH₄⁺ 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 Aₜ 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 pCO2, while NH4+ 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 NH4+ while estimating calcification rates based on A7 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 A7 have low analytical error and changes in A7 are twice larger than changes in Ca2+ (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 A7. 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 Ca2+ and Mg2+ need to be measured. Moreover, changes in Mg2+ remain difficult to precisely estimate in seawater due to high background concentrations and low incorporation rates.

Acknowledgements. We thank Zhimian Cao for help with the implementation of the analytical protocol for calcium measurements; Robinson Bordes-Mercier and Luuk van der Heijden for initial testing of the experimental and analytical protocols, and David Luquet, Didier Robin, Laurent Giletta and Stefano Schenone for diving and collecting the organisms. L.U. was supported by the Erasmus program Student Mobility for Placement, which enabled this collaboration. This work was funded by the French programme PNEC (Programme National Environnement Côtes, Institut National des Sciences de l’Univers), the EC FP7 projects European Project on Ocean Acidification (EPOCA; grant agreement 211384) and Mediterranean Sea Acidification in a changing climate (MedSeA; grant agreement 265103), and the BNP-Paribas Foundation.

LITERATURE CITED


Gattuso JP, Hansson L (2011) Ocean acidification: backgro...
Sokal RR, Rohlf FJ (1995) Biometry, the principles and practice of statistics in biological research, 3rd edn. WH Freeman, New York, NY

Editorial responsibility: James McClintock, Birmingham, Alabama, USA

Submitted: November 11, 2014, Accepted: March 17, 2015
Proofs received from author(s): April 16, 2015