Acidification, not carbonation, is the major regulator of carbon fluxes in the coccolithophore *Emiliania huxleyi*

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**Summary**
- A combined increase in seawater [CO$_2$] and [H$^+$] was recently shown to induce a shift from photosynthetic HCO$_3^-$ to CO$_2$ uptake in *Emiliania huxleyi*. This shift occurred within minutes, whereas acclimation to ocean acidification (OA) did not affect the carbon source.
- To identify the driver of this shift, we exposed low- and high-light acclimated *E. huxleyi* to a matrix of two levels of dissolved inorganic carbon (1400, 2800 μmol kg$^{-1}$) and pH (8.15, 7.85) and directly measured cellular O$_2$, CO$_2$ and HCO$_3^-$ fluxes under these conditions.
- Exposure to increased [CO$_2$] had little effect on the photosynthetic fluxes, whereas increased [H$^+$] led to a significant decline in HCO$_3^-$ uptake. Low-light acclimated cells overcompensated for the inhibition of HCO$_3^-$ uptake by increasing CO$_2$ uptake. High-light acclimated cells, relying on higher proportions of HCO$_3^-$ uptake, could not increase CO$_2$ uptake and photosynthetic O$_2$ evolution consequently became carbon-limited.
- These regulations indicate that OA responses in photosynthesis are caused by [H$^+$] rather than by [CO$_2$]. The impaired HCO$_3^-$ uptake also provides a mechanistic explanation for lowered calcification under OA. Moreover, it explains the OA-dependent decrease in photosynthesis observed in high-light grown phytoplankton.

**Introduction**

Coccolithophores are unicellular calcareous algae that take a dual role in global carbon cycling. During photosynthesis, carbon dioxide (CO$_2$) is fixed into organic matter, leading to a net decrease in dissolved inorganic carbon (DIC) and CO$_2$ from seawater. In the process of calcification, calcium carbonate (CaCO$_3$) is precipitated, which results in lowered DIC and alkalinity, thus elevated CO$_2$ levels. *Emiliania huxleyi* is the most abundant coccolithophore in the present-day ocean with a distribution from tropical to subpolar waters (Wintet et al., 2013). The species is able to form extensive blooms (Brown & Yoder, 1994; Sadeghi et al., 2012), which are often associated with a shallow mixed-layer depth and high irradiances (Nanninga & Tyrrell, 1996; Raitos et al., 2006). As one of the most important pelagic calcifiers, *E. huxleyi* has been a major focus of oceanographic research over the last decades, in particular with respect to ocean acidification (OA; e.g. Rost & Riebesell, 2004; Raven & Crawford, 2012).

As the ocean takes up anthropogenic CO$_2$, levels of HCO$_3^-$ and CO$_2$ increase, whereas pH and levels of CO$_3^{2-}$ decrease (Wolf-Gladrow et al., 1999). These changes in carbonate chemistry are often summarized as OA, but strictly speaking this phenomenon comprises carbonation (i.e. increased [CO$_2$] and [HCO$_3^-$]) as well as acidification (i.e. increased [H$^+$]/lowered pH). With a few exceptions, investigations of OA effects on *E. huxleyi* and other coccolithophores showed stimulated or unaffected production rates of particulate organic carbon (POC, i.e. biomass), with concomitantly impaired or unaffected production rates of particulate inorganic carbon (PIC, i.e. CaCO$_3$; see Raven & Crawford, 2012, for overview). Some of the observed diversity in the OA responses could be attributed to genetic variability; but more importantly environmental factors such as irradiance were shown to modulate OA effects (Nielsen, 1997; Zondervan et al., 2002; van de Poll et al., 2007; Feng et al., 2008; Rokitta & Rost, 2012; Sett et al., 2014; Xu & Gao, 2015). OA responses are typically measured after acclimation to altered conditions over several generations, allowing cells to adjust their metabolism. A study by Barcelos e Ramos et al. (2010) demonstrated that the OA-induced changes in cellular POC and PIC production are already evident after a few hours, indicating that OA effects are relatively immediate. In order to identify the drivers causing the OA responses in *E. huxleyi*, Bach and co-workers disentangled the effects of carbonation and acidification by acclimating cells to artificial carbonate chemistry conditions (Bach et al., 2011, 2013). In these experiments, POC and PIC production were shown to be stimulated by carbonation, but inhibited by acidification.

In order to improve our understanding of *E. huxleyi*’s response to OA, it is important to assess which cellular processes are affected by carbonation, acidification or the combination of both. *Emiliania huxleyi* is known to use CO$_2$ and HCO$_3^-$ as external inorganic carbon (Ci) sources of photosynthesis, but the estimated proportions of CO$_2$ uptake differ between studies and depend on the applied methods and assay conditions (e.g. Sikes
et al., 1980; Herfort et al., 2002; Trimborn et al., 2007; Kottmeier et al., 2014). The increase in POC production after acclimation to OA is often attributed to the higher aqueous CO2 levels, which are thought to directly increase the diffusive CO2 supply at the CO2-fixing enzyme Ribulose-1,5-bisphosphate-carboxylase/oxygenase (RubisCO; Raven & Johnston, 1991; Rokitta & Rost, 2012; Stojkovic et al., 2013). A recent study demonstrated that the fraction of photosynthetic CO2 uptake relative to active HCO3− uptake is indeed strongly increased under high [CO3]/low pH (Kottmeier et al., 2014). This switch in the Ci source occurred at short timescales of seconds to minutes, whereas the acclimation to OA did not significantly affect the Ci source. Thus, the beneficial OA effect seems to be directly caused by the changing carbonate chemistry rather than by changes in the expression of genes related to the CO2-concentrating mechanism (CCM). The inhibitory effect of OA on PIC production is often attributed to changes in electrochemical gradients under high [H+] and the associated costs of H+ removal (Anning et al., 1999; Betry et al., 2002; Suffrian et al., 2011; Taylor et al., 2011). Tracer studies found HCO3− to be the major external Ci source for calcification (Paasche, 1964; Sikes et al., 1980; Buitenhuis et al., 1999; Herfort et al., 2002; Rost et al., 2002), and it was suggested that increased H+ levels also affect HCO3− uptake mechanisms (Fukuda et al., 2014). Despite the gained knowledge on cellular processes, relatively little is known about the differential effects of carbonation and acidification on photosynthesis, calcification and their underlying Ci supply.

In order to investigate the drivers causing the immediate shifts in the photosynthetic Ci source under high [CO3]/low pH (Kottmeier et al., 2014), here we measured the photosynthetic oxygen (O2) and Ci fluxes in direct response to carbonation, acidification and the combination of both. To this end, we acclimated both life-cycle stages of *Emiliania huxleyi* to present-day carbonate chemistry and exposed them to a matrix of two DIC levels (1400 and 2800 μmol kg−1) and two pH values (8.15 and 7.85), yielding three different CO2 concentrations (~10, 20 and 40 μmol kg−1; Fig. 1). To further address the effect of energization, cells were acclimated to low and high photon flux densities (PFD; 50 and 400 μmol photons m−2 s−1), and fluxes were measured at two different PFD (180 and 700 μmol photons m−2 s−1).

**Materials and Methods**

**Culture conditions**

The calcifying diplont (diploid life-cycle stage) *Emiliania huxleyi* (Lohmann) Hay and Mohler, strain RCC 1216, and its noncalcifying haplont, RCC 1217, were acclimated to low and high light levels (LL, 50 ± 30 μmol photons s−1 m−2; HL, 400 ± 30 μmol photons s−1 m−2) under present-day carbonate chemistry and a 16 h:8 h, light:dark-cycle. Light was provided by daylight lamps (FQ 54W/965HO; OSRAM, Munich, Germany) and adjusted by measuring photon flux densities (PFD) inside water-containing culturing bottles with a Walz Universal Light meter (ULM 500; Walz, Effeltrich, Germany) using a 4π-sensor (US-SQS/L).

Cells were grown as dilute-batch cultures in sterile-filtered North Sea seawater (0.2 μm, Sartobran 300; Sartorius AG, Göttingen, Germany) enriched with phosphate and nitrate (~7 and ~100 μmol kg−1, respectively) as well as vitamins and trace metals according to F/2 (Guillard and Ryther, 1962). Culturing was performed in sterilized, gas-tight 2-L borosilicate bottles (Duran Group, Mainz, Germany), which were placed on roller tables to enable an homogenous cell suspension. Growth temperature was 15 ± 2°C and was monitored by an Almemo 28–90 data logger (Ahlborn, Holzkirchen, Germany).

In all treatments, acclimations were performed under a CO2 partial pressure (pCO2) of 380 μatm (38.5 Pa), representing near-present-day conditions. The pCO2 was adjusted by pre-aerating culture media with humidified, 0.2-μm-filtered air (Midisart 2000, PTFE; Sartorius AG), containing the desired pCO2. The gas mixture was created by a gas flow controller (CGM 2000; MCZ Umwelttechnik, Bad Nauheim, Germany) using pure CO2 (Air Liquide, Düsseldorf, Germany) and CO2-free air (Air purification system; Parker, Kaarst, Germany). During the acclimation, head space inside the culture bottles was minimized to avoid outgassing effects. Carbonate chemistry was monitored based on total alkalinity (TA) measurements by potentiometric titration (Dickson, 1981; TitroLine alpha plus, measurement reproducibility ± 7 μmol kg−1; Schott Instruments, Mainz, Germany) and colorimetric DIC measurements with a QuAAtro autoanalyzer (measurement reproducibility ± 5 μmol kg−1; Seal Analytical, Norderstedt, Germany) in sterile-filtered samples with the method of Stoll et al. (2001). Calculations of the carbonate system (CO2sys; Pierrot et al., 2006) were based on TA and DIC (Supporting Information Table S1). To monitor potential drifts of the carbonate chemistry on a daily basis, potentiometric measurements of pHNBS were performed with a Metrohm pH meter (826 pH mobile; Metrohm, Filderstadt, Germany) with an electrode containing an integrated temperature sensor (Aquatron Plus with Pt 1000, measurement reproducibility ± 0.1 pH units).

Cell growth was monitored by daily cell counting with a Coulter Counter (Beckman-Coulter, Fullerton, CA, USA) and specific
growth constants $\mu$ (d$^{-1}$) were determined as $\mu = (\log_{e} c_{1} - \log_{e} c_{0}) / \Delta t$ (where $c_{1}$ and $c_{0}$ are cell concentrations (cells ml$^{-1}$); $\Delta t$, time interval (d)). In both life-cycle stages, $\mu$ was more or less equal and significantly reduced in the low-light acclimations ($0.7$ d$^{-1}$), confirming a light limitation in this treatment (Table S1). In the high-light treatment, $\mu$ ($1.1$ d$^{-1}$) was at the upper range of previously reported growth constants for the same strain (Langer et al., 2009; Rokitta & Rost, 2012).

Mass spectrometric flux measurements

Photosynthetic and respiratory $O_{2}$ and $C_{i}$ fluxes were measured with a mass spectrometer (Isoprime, GV Instruments, Manchester, UK) that was coupled to a cuvette via a gas-permeable PTFE membrane (0.01 mm). This membrane-inlet mass spectrometry (MIMS) technique uses the chemical disequilibrium between $CO_{2}$ and $HCO_{3}^{-}$ during steady-state photosynthesis to distinguish $CO_{2}$ and $HCO_{3}^{-}$ uptake across the plasmalemma. Estimates of these fluxes were made following the equations of Badger et al. (1994). To include the process of calcification, we added DIC was quantitatively converted to $CO_{2}$. Baseline values were obtained by adding sodium hydroxide (0.25 mmol l$^{-1}$) into DIC-free media, ensuring that any residual DIC was converted to $CO_{3}^{2-}$. Calibration for $[O_{2}]$ was obtained by equilibrating medium with air (21% $O_{2}$), followed by the addition of sufficient amounts of sodium dithionite (Merck) to quantitatively scavenge $O_{2}$ (0% $O_{2}$). MIMS signals were translated into $[O_{2}]$ by applying the $O_{2}$ solubility constants of seawater (Weiss, 1970). All $O_{2}$ signals were furthermore corrected for the machine-inherent consumption.

Experiments were performed with cells in their exponential growth phase with maximal cell concentrations of $5 \times 10^4$ cells ml$^{-1}$ in 6–10 h after the start of the light period. Before the measurements, cells were concentrated to $4 \times 10^7$ cells ml$^{-1}$ at acclimation temperature by gentle vacuum filtration over polycarbonate filters (Isopore TSTP, 3 μm or RTTP, 1.2 μm; Isopore membranes, Merck, Darmstadt, Germany). In this process, the medium was successively exchanged with pH-buffered DIC-free culture medium (50 mM N,N-bis(2-hydroxyethyl)-glycine, BICINE; pH$_{NBS}$ of 7.85 or 8.15, and 8 ml were placed into an temperature-controlled MIMS cuvette in the dark. Subsequently, 25 μmol kg$^{-1}$ membrane-impermeable dextrane-bound sulfonamide (DBS; Synthelec, Lund, Sweden) was added, inhibiting any external carbonic anhydrase. Samples were continuously stirred to keep the cell suspension homogenously mixed. To disentangle carbonate chemistry in the cuvette, inorganic carbon was added as ~1400 or ~2800 μmol kg$^{-1}$ $NaHCO_{3}$ to the DIC-free medium, buffered at a pH of 8.15 or 7.85, yielding four different carbonate chemistry conditions (Fig. 1; Table 1): 'Low DIC/High pH' (LDICHpH), 'High DIC/High pH' (HDICHpH), 'Low DIC/Low pH' (LDICLpH), and 'High DIC/Low pH' (HDICLpH). For each carbonate chemistry condition, photosynthetic and respiratory $O_{2}$ and $C_{i}$ fluxes were measured in consecutive light-dark intervals (6 min per step), at two different light levels (180 and 700 μmol photons m$^{-2}$ s$^{-1}$).

Calculations of oxygen and carbon fluxes

**Oxygen fluxes** Net photosynthesis ($Phot$, μmol kg$^{-1}$ min$^{-1}$) and respiration ($Resp$, μmol kg$^{-1}$ min$^{-1}$) were deduced from steady-state $O_{2}$ fluxes in the light and dark, respectively (Badger et al., 1994):

$$ \text{Phot} = \frac{dO_{2}}{dt} \quad \text{Eqn 1} $$

$$ \text{Resp} = -\frac{dO_{2}}{dt} \quad \text{Eqn 2} $$

**Carbonate chemistry before light (BL)** For the calculation of the $C_{i}$ fluxes, carbonate chemistry before and after the light phase was determined by mass spectrometry using a MIMS technique (Badger et al., 1994).

### Table 1 Carbonate chemistry during mass measurements of $O_{2}$ and inorganic carbon ($C_{i}$) fluxes in *Emiliania huxleyi*

<table>
<thead>
<tr>
<th>Acclimation</th>
<th>Carbonate chemistry</th>
<th>$L_{DIC,HpH}$</th>
<th>$H_{DIC,HpH}$</th>
<th>$L_{DIC,LpH}$</th>
<th>$H_{DIC,LpH}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2N LL</td>
<td>$[CO_{2}]$</td>
<td>12.4 ± 0.6</td>
<td>22.0 ± 0.8</td>
<td>24.0 ± 1.5</td>
<td>43.7 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>$[HCO_{3}^{-}]$</td>
<td>1370 ± 70</td>
<td>2500 ± 90</td>
<td>1420 ± 80</td>
<td>2590 ± 110</td>
</tr>
<tr>
<td></td>
<td>$[H+]$</td>
<td>9.5 ± 0.2</td>
<td>9.5 ± 0.2</td>
<td>18.4 ± 0.3</td>
<td>18.4 ± 0.3</td>
</tr>
<tr>
<td>2N HL</td>
<td>$[CO_{2}]$</td>
<td>13.6 ± 0.9</td>
<td>22.7 ± 1.0</td>
<td>nd</td>
<td>47.4 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>$[HCO_{3}^{-}]$</td>
<td>1560 ± 110</td>
<td>2730 ± 130</td>
<td>nd</td>
<td>2730 ± 110</td>
</tr>
<tr>
<td></td>
<td>$[H+]$</td>
<td>9.1 ± 0.3</td>
<td>8.9 ± 0.3</td>
<td>nd</td>
<td>18.8 ± 0.3</td>
</tr>
<tr>
<td>1N LL</td>
<td>$[CO_{2}]$</td>
<td>10.6 ± 0.5</td>
<td>20.5 ± 0.3</td>
<td>17.1 ± 0.5</td>
<td>40.9 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>$[HCO_{3}^{-}]$</td>
<td>1200 ± 50</td>
<td>2400 ± 30</td>
<td>1050 ± 30</td>
<td>2460 ± 160</td>
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<tr>
<td></td>
<td>$[H+]$</td>
<td>9.2 ± 0.1</td>
<td>9.2 ± 0.1</td>
<td>17.4 ± 0.5</td>
<td>18.1 ± 0.0</td>
</tr>
<tr>
<td>1N HL</td>
<td>$[CO_{2}]$</td>
<td>10.5 ± 0.6</td>
<td>18.6 ± 1.4</td>
<td>17.9 ± 1.8</td>
<td>32.5 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>$[HCO_{3}^{-}]$</td>
<td>1150 ± 70</td>
<td>2160 ± 130</td>
<td>1090 ± 120</td>
<td>1940 ± 200</td>
</tr>
<tr>
<td></td>
<td>$[H+]$</td>
<td>9.3 ± 0.1</td>
<td>9.2 ± 0.1</td>
<td>17.5 ± 0.4</td>
<td>18.1 ± 0.5</td>
</tr>
</tbody>
</table>

Concentrations of $CO_{2}$ (μmol kg$^{-1}$), $HCO_{3}^{-}$ (μmol kg$^{-1}$) and $H^{+}$ (nmol kg$^{-1}$) were assessed by means of mass spectrometry ($n = 3$; ± SD).

2N LL/HL, diploid life-cycle stage acclimated to low/high light; 1N LL/HL, haploid life-cycle stage acclimated to low/high light; $L_{DIC,HpH}$, low dissolved inorganic carbon (DIC)/high pH; $H_{DIC,HpH}$, high DIC/high pH; $L_{DIC,LpH}$, low DIC/low pH; $H_{DIC,LpH}$, high DIC/low pH; nd, not determined.
was determined. \([\text{CO}_2]_{\text{BL}}\) (\(\text{mol} \text{ kg}^{-1}\)) could be directly taken from measured signals, whereas \([\text{HCO}_3^-]_{\text{BL}}\) (\(\text{mol} \text{ kg}^{-1}\)) was calculated according to Badger et al. (1994):

\[
[HCO_3^-]_{\text{BL}} = \frac{d[CO_2]_{\text{BL}}}{dt} + k_+ [CO_2]_{\text{BL}} - \frac{\text{Resp/RQ}}{k_-} \tag{Eqn 3}
\]

(d\(CO_2/dt\)) steady-state CO\(_2\) evolution in the dark (\(\text{mol} \text{ kg}^{-1} \text{ min}^{-1}\)); \(k_+\) and \(k_-\), effective rate constants for the conversion of \(\text{CO}_2\) to \(\text{HCO}_3^-\) (\(\text{mol} \text{ kg}^{-1} \text{ min}^{-1}\)) and vice versa; RQ, respiratory quotient of 1 (Burkhardt et al., 2001; Rost et al., 2007)). Following Schulz et al. (2007), we applied the calculated effective rate constants derived from the measured pH, temperature and salinity in our assays:

\[
k_+ = k_+ [H^+] + k_- \tag{Eqn 4}
\]

\[
k_+ = k_{+1} + k_{+4} [OH^-] \tag{Eqn 5}
\]

\([H^+]\) and \([OH^-]\), concentrations of hydrogen and hydroxide ions, respectively (\(\text{mol} \text{ kg}^{-1}\)); \(k_{+1}\), \(k_{+4}\), \(k_-\) and \(k_{+4}\), rate constants (Zeebe & Wolf-Gladrow, 2001). To assess \([H^+]\), known \([\text{DIC}]\) (\(\text{mol} \text{ kg}^{-1}\)) was added to cell-free medium. From the resulting increase in \([\text{CO}_2]\) (\(\text{mol} \text{ kg}^{-1}\)), the ratio of \([\text{DIC}] : \text{CO}_2\) and thus \([H^+]\) could be derived (Zeebe & Wolf-Gladrow, 2001):

\[
[H^+] = \frac{-K^* [CO_2] - \sqrt{(K^* [CO_2])^2 - 4(\text{[DIC]} K^* K^*)}}{2(\text{[CO}_2] - \text{[DIC]})} \tag{Eqn 6}
\]

\((K^*\) and \(K^*\), stoichiometric equilibrium constants (Roy et al., 1993)). \([\text{DIC}]_{\text{BL}}\) was derived as the sum of the C\(_4\) species, where carbonate ions (\(\text{[CO}_3^{2-}]\)) can be assumed to be in equilibrium with \(\text{[HCO}_3^-]\) (Schulz et al., 2006):

\[
[\text{DIC}]_{\text{BL}} = [\text{CO}_2]_{\text{BL}} + (1 + r)[\text{HCO}_3^-]_{\text{BL}} \tag{Eqn 7}
\]

The constant \(r\) hereby represents the pH-dependent ratio between \(\text{[HCO}_3^-]\) and \(\text{[CO}_3^{2-}]\) (Zeebe & Wolf-Gladrow, 2001; Schulz et al., 2007), which is defined as:

\[
r = \frac{\text{[CO}_3^{2-}]}{[\text{HCO}_3^-]} = \frac{K^*}{[H^+]} \tag{Eqn 8}
\]

**Carbonate chemistry at the end of light (EL)** \([\text{CO}_2]_{\text{EL}}\) (\(\text{mol} \text{ kg}^{-1}\)) was directly obtained from measurements, whereas \([\text{HCO}_3^-]_{\text{EL}}\) (\(\text{mol} \text{ kg}^{-1}\)) was derived following Schulz et al. (2007):

\[
[\text{HCO}_3^-]_{\text{EL}} = \frac{([\text{DIC}]_{\text{BL}} - [\text{DIC}]_{\text{consumed}} - [\text{CO}_2]_{\text{EL}})/(1 + r)}{1 + r} \tag{Eqn 9}
\]

[\text{DIC}]_{\text{consumed}} \text{ represents the concentration of DIC that was consumed in the course of the light interval and was defined as the sum of DIC used for photosynthesis ((O\(_2\))_{\text{evolved}}/PQ, \(\text{mol} \text{ kg}^{-1}\)) and for calcification ([DIC]_{\text{CaCO}_3}, \(\text{mol} \text{ kg}^{-1}\)):

\[
[\text{DIC}]_{\text{consumed}} = \frac{[O_2]_{\text{evolved}}}{PQ} + [\text{DIC}]_{\text{CaCO}_3} \tag{Eqn 10}
\]

\([O_2]_{\text{evolved}}\) hereby represents the concentration of \(O_2\) that evolved over the course of the light phase and PQ is the photosynthetic quotient of 1.1 (Burkhardt et al., 2001; Rost et al., 2007). \([\text{DIC}]_{\text{CaCO}_3}\) was constrained by the measured ratio of particulate inorganic to organic carbon (PIC : POC) of the calcifying diploid life-cycle stage under similar light treatments (1.4 at low-light and 0.8 at high-light acclimations; Rokitta & Rost, 2012) and was assumed to scale linearly with \([O_2]_{\text{evolved}}\) (e.g. Paasche, 1999). Additionally, calcite production was normalized to the photoperiod (Schulz et al., 2007):

\[
[\text{DIC}]_{\text{CaCO}_3} = \frac{[O_2]_{\text{evolved}}}{PQ} \times \frac{16 \text{ Phot} - 8 \text{ Resp} \times \text{PIC}}{16 \text{ Phot} \times \text{POC}} \tag{Eqn 11}
\]

In the noncalcifying haploid stage, PIC : POC was set to zero. Sensitivity analyses in which the PIC : POC were allowed to vary within typical uncertainties, revealed negligible effects on the calculated carbonate chemistry and photosynthetic fluxes.

**Carbon fluxes** Knowing the carbonate chemistry, total net CO\(_2\) uptake \((\text{CO}_2)_{\text{up-total}}\) (\(\text{mol} \text{ kg}^{-1} \text{ min}^{-1}\)) was inferred directly from the steady-state CO\(_2\) drawdown in the light following Badger et al. (1994):

\[
\text{CO}_2_{\text{up-total}} = -\frac{d\text{CO}_2}{dt}_{\text{EL}} - k_+ [\text{CO}_2]_{\text{EL}} + k_- [\text{HCO}_3^-]_{\text{EL}} \tag{Eqn 12}
\]

Total CO\(_2\) uptake can be divided into one part used for photosynthesis \((\text{CO}_2)_{\text{up-PS}}\) (\(\text{mol} \text{ kg}^{-1} \text{ min}^{-1}\)) and another part used for calcification \((\text{CO}_2)_{\text{up-CaCO}_3}\) (\(\text{mol} \text{ kg}^{-1} \text{ min}^{-1}\)). As \(\text{HCO}_3^-\) is the major external C\(_4\) source for calcification, we assumed that only 20% of calcification is supplied by external CO\(_2\) (Sikes et al., 1980; Paasche, 2001; Rost et al., 2002). Overall calcification was constrained by photoperiod-normalized PIC : POC ratios and was assumed to scale linearly with the photosynthetic oxygen evolution:

\[
\text{CO}_2_{\text{up-CaCO}_3} = 0.2 \times \frac{\text{Phot}}{\text{POC}} \times \frac{\text{PIC}}{\text{POC}} \times \frac{16 \text{ Phot} - 8 \text{ Resp}}{16 \text{ Phot}} \tag{Eqn 13}
\]

Please note that, similar to PIC : POC ratios, errors in the assumption of the CO\(_2\) usage for calcification can affect the estimated photosynthetic fluxes by relative constant and small offsets, but do not change the overall observed regulation patterns in response to carbonate chemistry. Accounting for the CO\(_2\) uptake for calcification, \(\text{CO}_2_{\text{up-PS}}\) could be calculated as:
CO$_2$up$_{PS}$ = CO$_2$up$_{total}$ − CO$_2$up$_{CaCO_3}$

Eqn 14

Photosynthetic HCO$_3^-$ uptake (HCO$_3^-$up, μmol kg$^{-1}$ min$^{-1}$) was estimated as the difference between photosynthetic net C$_i$ fixation (calculated as Phot PQ$^{-1}$) and net CO$_2$ uptake for photosynthesis:

\[ \text{HCO}_3^\text{up} = \frac{\text{Phot}}{\text{PQ}} - \text{CO}_2\text{up}_{PS} \]

Eqn 15

Knowing the photosynthetic net CO$_2$ uptake, its fraction of the overall net photosynthetic C$_i$ uptake ($f_{CO_2}$; cf. Kottmeier et al., 2014) was derived as:

\[ f_{CO_2} = \frac{\text{CO}_2\text{up}_{PS}}{\text{Phot}} \]

Eqn 16

Rate normalization All rates were normalized to the amount of chlorophyll $a$ (Chl$a$) in the concentrated samples. Known amounts of cell suspension were filtered onto cellulose nitrate filters (0.45 μm; Sartorius, Gottingen, Germany) that were instantly frozen in liquid nitrogen. After extraction in 90% acetone, Chl$a$ content was determined fluorimetrically (TD-700 fluorometer; Turner Designs, Sunnyvale, CA, USA) following the protocol of Knap et al. (1996).

Statistics All experiments were carried out in biological triplicates. Fluxes estimated for the different carbonate chemistry conditions and at the same incoming PFD were tested pairwise for significant differences applying two-sided $t$-tests. Effects were called significant when $P$-values were ≤ 0.05. In the figures, such significant differences were indicated by different lower-case characters (e.g., a and b). Values denoted by two letters (e.g., ab) represent data that are not significantly different from a or b.

Results

In the following, we describe treatment-specific differences in short-term responses to altered carbonate chemistry and light. For clarity, only the fluxes of the diplont are shown in Figs 2, 3. Fluxes of the haplont are given in Table 2.

Oxygen fluxes

In both life-cycle stages and light acclimations, net photosynthesis increased under increasing incoming light (Fig. 2a,b; PFD 180 vs 700), whereas dark respiration was generally independent of the light levels applied before the dark phase (Fig. 2c,d). The dependency on carbonate chemistry was stage and acclimation-light specific (Fig. 2a–d; Table 2).

In the diplont acclimated to low light (2N LL), net photosynthesis was significantly stimulated under combined carbonation and acidification (HDICLpH; Fig. 2a). This increase could not be attributed exclusively to carbonation or acidification, but appeared to be a product of both. Respiration in 2N LL decreased under HDICLpH (significantly only at PFD 180). This effect seemed to be driven by acidification, because the rates decreased significantly under both low-pH conditions, but not with carbonation (Fig. 2c).

In the diplont acclimated to high light (2N HL), net photosynthesis was significantly impaired under H$_{DICLpH}$ (Fig. 2b).

Fig. 2 Short-term modulations in photosynthetic and respiratory O$_2$ fluxes of Emiliania huxleyi in response to low dissolved inorganic carbon (DIC)/high pH ($D_{DICLpH}$, white bars), as well as carbonation ($H_{DICLpH}$, dashed, light grey bars), acidification ($L_{DICLpH}$, light grey bars) and the combination of both ($H_{DICLpH}$, dashed, dark grey bars): Chl$a$-normalized photosynthetic net O$_2$ evolution (Phot; a, b) and respiration (Resp; c, d) were measured at low and high photon flux densities (PFD: 180 and 700 μmol photons m$^{-2}$ s$^{-1}$). Data are shown for the diploid life-cycle stage acclimated to low and high light (2N LL, 2N HL). Note: in 2N HL, no data for the L$_{DICLpH}$ condition were obtained. Error bar indicate mean ± SD ($n = 3$). Different lower-case characters indicate significant differences between the fluxes obtained at different carbonate chemistry conditions and same PFD.
Also this effect seemed to be caused by carbonation and acidification together, although the drivers could not be identified statistically due to the lack of the LDICLpH data (Fig. 1; Tables 1, 2). Respiration in 2N HL was largely unaffected by carbonate chemistry (Fig. 2d).

In contrast to the diplont, photosynthesis and respiration in the low- and high-light acclimated haplont (1N LL, 1N HL) were insensitive to the applied carbonate chemistry (Table 2).

Carbon fluxes

In both life-cycle stages and light acclimations, the higher Ci demands imposed by the higher incoming light levels during measurements were in most cases covered by additional HCO$_3^-$ uptake, whereas photosynthetic net CO$_2$ uptake was largely unaffected by the incoming light (Fig. 3a–d; Table 2; PFD 180 vs PFD 700). The dependency of Ci fluxes on carbonate chemistry was clearly stage and acclimation-light specific (Fig. 3; Table 2).

In 2N LL, the photosynthetic net CO$_2$ uptake increased significantly under HDICLpH at both applied light levels, and these higher fluxes seemed to be driven mainly by acidification because CO$_2$ uptake was strongly increased under both low-pH conditions (Fig. 3a). Carbonation at high pH did not stimulate the CO$_2$ uptake, whereas carbonation at low pH (LDICLpH vs HDICLpH) additionally increased CO$_2$ uptake. HCO$_3^-$ uptake in 2N LL decreased significantly under HDICLpH (Fig. 3c). This decrease was clearly driven by acidification because HCO$_3^-$ uptake was decreased under both low-pH conditions, independent of carbonation. The described opposing short-term regulation of CO$_2$ and HCO$_3^-$ uptake under HDICLpH caused significant shifts in f$_{CO2}$ from ~0.3 to ~0.9 (Fig. 3e).

In 2N HL, photosynthetic net CO$_2$ uptake was relatively unaffected by carbonate chemistry (Fig. 3b). Similar to the low-light acclimated cells, HCO$_3^-$ uptake for photosynthesis in 2N HL decreased significantly under HDICLpH, presumably also driven by acidification (Fig. 3d). As a consequence of the relatively constant net CO$_2$ uptake and the decreased HCO$_3^-$ uptake, f$_{CO2}$ increased significantly from ~0.2 to ~0.7 at a PFD of 180, whereas the increase was insignificant at 700 µmol photons m$^{-2}$ s$^{-1}$ (Fig. 3f).

In 1N LL, photosynthetic net CO$_2$ uptake was close to zero and the photosynthetic HCO$_3^-$ uptake clearly dominated the Ci fluxes (Table 2). Both CO$_2$ and HCO$_3^-$ fluxes were unaffected by carbonate chemistry, resulting in constant and low f$_{CO2}$ values (~0.1 on average; Table 2). In 1N HL, photosynthetic net CO$_2$ uptake was negative, reflecting a net CO$_2$ efflux alongside a high HCO$_3^-$ uptake (Table 2). Also here, CO$_2$ and HCO$_3^-$ fluxes were not significantly affected by carbonate chemistry.

Fig. 3 Short-term modulations in external inorganic carbon (Ci) fluxes of *Emiliania huxleyi* in response to low dissolved inorganic carbon (DIC)/high pH (LDICLpH; white bars), as well as carbonation (HDICLpH; dashed, light grey bars), acidification (LDIC$	ext{H}^+$; light grey bars) and the combination of both (HDICLpH; dashed, dark grey bars): Chl-a-normalized photosynthetic net CO$_2$ uptake (CO$_2$uptPS; a, b), photosynthetic HCO$_3^-$ uptake (HCO$_3^-$ uptPS; c, d) and the fraction of overall photosynthetic net Ci uptake that is covered by net CO$_2$ uptake (f$_{CO2}$; e, f) were measured at low and high photon flux densities (PFD; 180 and 700 µmol photons m$^{-2}$ s$^{-1}$). Data are shown for the diploid life-cycle stage acclimated to low and high light (2N LL, 2N HL). Note: in 2N HL, no data for the LDICLpH condition were obtained. Error bar indicate mean ± SD (n = 3). Different lower-case characters indicate significant differences between the fluxes obtained at different carbonate chemistry conditions and same PFD.
Table 2 Short-term modulations in photosynthetic O₂ and inorganic carbon (Cᵢ) fluxes of *Emiliania huxleyi* in response to low dissolved inorganic carbon (DIC) and high pH (LDICLₚH), as well as carbonation (HDICₚH), acidification (LDICLₚH) and the combination (HDICLₚH)

<table>
<thead>
<tr>
<th>Acclimation</th>
<th>PFD</th>
<th>Carbonate chemistry</th>
<th>Phot (μmol mg⁻¹ h⁻¹)</th>
<th>Resp (μmol mg⁻¹ h⁻¹)</th>
<th>CO₂uptPS (μmol mg⁻¹ h⁻¹)</th>
<th>HCO₃⁻uptPS (μmol mg⁻¹ h⁻¹)</th>
<th>fCO₂</th>
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<tbody>
<tr>
<td>2N LL</td>
<td>180</td>
<td>LDICLₚH</td>
<td>217 ± 17</td>
<td>75 ± 7</td>
<td>51 ± 20</td>
<td>147 ± 15</td>
<td>0.26 ± 0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HDICₚH</td>
<td>236 ± 34</td>
<td>63 ± 9</td>
<td>58 ± 19</td>
<td>156 ± 43</td>
<td>0.28 ± 0.11</td>
</tr>
<tr>
<td></td>
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<td>LDICₚH</td>
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<td></td>
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<td>HDICₚH</td>
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<tr>
<td>700</td>
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<td>327 ± 5</td>
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<td>23 ± 39</td>
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</table>

Chl-a normalized photosynthetic net O₂ evolution (Phot) and respiration (Resp), photosynthetic net CO₂ uptake (CO₂uptPS), photosynthetic HCO₃⁻ uptake (HCO₃⁻uptPS) and the fraction of overall photosynthetic net Cᵢ uptake that is covered by net CO₂ uptake (fCO₂) were measured at low and high photon flux densities (PFD; 180 vs 700 μmol photons m⁻² s⁻¹; n = 3; ± SD). 2N LL/HL, diploid life-cycle stage acclimated to low light/high light; 1N LL/HL, haploid life-cycle stage acclimated to low/high light; LDICLₚH, low DIC/high pH; HDICₚH, high DIC/high pH; LDICₚH, low DIC/low pH; HDICₚH, high DIC/low pH; nd, not determined.

were unaffected by carbonate chemistry, resulting in constant and negative fCO₂ values (~0.1).

**Discussion**

In this study, we investigated *Emiliania huxleyi*’s photosynthetic O₂ and Cᵢ fluxes and their short-term modulations in response to changing carbonate chemistry and light. In the diploid life-cycle stage (diploïd), cellular fluxes were shown to be highly sensitive and to rapidly respond to the applied conditions. In the haploid stage (haploïd), cellular fluxes were rather constant, even across large changes in carbonate chemistry.

**H⁺-driven increase in CO₂ uptake stimulates photosynthesis in low-light acclimated diploïds**

In the low-light acclimated diploid (2N LL), rates of photosynthetic O₂ evolution were in a similar range as measured earlier under comparable conditions (Nielsen, 1995; Rokitta & Rost, 2012). They stayed relatively constant under carbonation or acidification alone, but were strongly stimulated by combined carbonation and acidification (Fig. 2a). Ocean acidification (OA) has earlier been shown to affect cellular fluxes rapidly (Barcelos e Ramos et al., 2010; Kottmeier et al., 2014). An immediate stimulation in photosynthesis, if maintained over longer timescales, could therefore also explain the increase in particulate organic carbon (POC) production that is typically observed in OA-acclimated coccolithophores (Raven & Crawford, 2012). Even though the applied carbonate chemistry matrix generally allowed for the distinction between the effects of carbonation and acidification, their differential effects were not evident from the observed O₂ fluxes (Fig. 2a). Only by measuring the underlying Cᵢ acquisition was it possible to identify the drivers behind the photosynthetic responses (Fig. 3a,c): Net CO₂ uptake was strongly promoted under acidification as well as under combined carbonation and acidification (Fig. 3a), whereas HCO₃⁻ uptake...
was strongly downscaled under these conditions (Fig. 3c). As the stimulation in net CO₂ uptake under combined carbonation and acidification exceeded the impairing effect on HCO₃⁻ uptake, the overall photosynthetic Cᵢ uptake and consequently photosynthetic O₂ evolution were increased under these conditions (Fig. 2a).

The transition from the active HCO₃⁻ to diffusive CO₂ uptake under short-term acidification is in line with Kottmeier et al. (2014), who observed that E. huxleyi increases the relative fraction of CO₂ usage when being exposed to high [CO₂]/low pH over short timescales. Here we show that this shift is caused by a combination of increased CO₂ uptake and decreased HCO₃⁻ uptake. The increased CO₂ usage is likely to decrease the energy demand of the cell, because transport of HCO₃⁻ is considered more costly due to the molecule’s negative charge and the large hydration envelope, properties that require an active transport (Burkhart et al. 2001; Beardall & Raven, 2004; Holtz et al., 2015b). Indeed, we found stimulated photosynthesis and decreased respiratory rates under acidification despite the same incoming light (Fig. 2a,c), indicating not only a more efficient CO₂ supply at RubisCO, but also altered energy allocations under these conditions. Such energy reallocations under OA have earlier been attributed to shifts from reductive towards oxidative pathways (Rokitta et al., 2012).

The H⁺-driven stimulation in CO₂ uptake contradicts the ‘fertilizing effect’ of CO₂ that is typically ascribed to OA. Contrary to the common notion that CO₂ uptake for photosynthesis benefits from carbonation, it was here promoted mainly by acidification, at least over the short timescales applied. The higher CO₂ uptake under combined carbonation and acidification compared to acidification alone indicated that high H⁺ levels generally increase the cellular CO₂ uptake capacity. Yet, the higher CO₂ availability was able to stimulate its uptake even further – carbonation and acidification acted synergistically. The H⁺-driven decrease in cellular HCO₃⁻ uptake, which occurred independent of the applied dissolved inorganic carbon (DIC) levels, indicated that the HCO₃⁻ transport capacity is generally downscaled under acidification. Carbonation alone had no effect on HCO₃⁻ uptake, suggesting that the transporters are substrate-saturated at the applied [HCO₃⁻] (~1300 and 2600 μmol kg⁻¹). This is in line with a study by Rost et al. (2006) who measured the short-term DIC-dependency of photosynthesis at constant pH and showed that HCO₃⁻ uptake in E. huxleyi was substrate-saturated even below [HCO₃⁻] of ~500 μmol kg⁻¹.

The H⁺-dependent regulations in Cᵢ fluxes are likely to be similar after acclimation. Bach et al. (2011), for instance, acclimated E. huxleyi to carbonate chemistry conditions in which either CO₂ or pH varied independently. They could show that PIC production increases with DIC if pH is buffered to ~8.0, but it decreases with increasing DIC if pH decreases concomitantly. This suggests that the negative H⁺ effects on HCO₃⁻ uptake are retained after acclimation. However, in contrast to our study, where no short-term carbonation effects were measured, Bach and coworkers found stimulated PIC and particulate inorganic carbon (PIC) production after acclimation to carbonation. Consequently, the cells were able to increase their Cᵢ uptake when being exposed to these conditions over longer timescales, possibly by expressing more HCO₃⁻ transporters. In order to examine how acclimation affects the sensitivity towards changing carbonate chemistry, future studies should investigate which short-term effects manifest over longer timescales.

The strong H⁺ effects on CO₂ and HCO₃⁻ uptake rates, observed in the current study, must originate from processes at the cell membrane or inside the cell, such as electrochemical gradients, enzyme activities and Cᵢ speciation (Mackinder et al., 2010; Suffrian et al., 2011; Taylor et al., 2011). The stimulated net CO₂ uptake under acidification could, for example, be explained by pH-dependent differences in membrane morphology (Leung et al., 2012), which may also affect the CO₂ permeability. It could also be caused by pH-dependent regulations of intracellular fluxes, for instance due to different enzyme activities, which may lead to a stronger inward CO₂ gradient. The decreased HCO₃⁻ uptake under acidification is apparently caused by a direct H⁺-driven inhibition of HCO₃⁻ transporters at the plasmalemma or chloroplast membrane. The diplont E. huxleyi expresses AE1 and AE2-type Cl⁻/HCO₃⁻ transporters of the Solute Carrier 4 (SLC4) family (Herfort et al., 2002; von Dassow et al., 2009; Mackinder et al., 2011; Rokitta et al., 2011; Bach et al., 2013). This enzyme family is well investigated in the context of renal acid/base regulation in mammals, where the activity of the anion exchangers has indeed been shown to be modulated by pH (Alper, 2006).

H⁺-driven decrease in HCO₃⁻ uptake causes carbon-limitation in high-light acclimated diplonts

In high-light acclimated diploid cells (2N HL), photosynthesis was inhibited under combined carbonation and acidification (Fig. 2b). This finding seems puzzling at first because in low-light acclimated cells (2N LL), the same carbonate chemistry had a pronounced beneficial effect on photosynthesis (Fig. 2a). However, light-dependent modulations in the sensitivity towards carbonate chemistry are well in line with other studies (e.g. Kranz et al., 2010; Gao et al., 2012; Rokitta & Rost, 2012; Jin et al., 2013; Hoppe et al., 2015). Rokitta & Rost (2012), for example, found that PIC production in E. huxleyi is strongly stimulated when acclimated to OA and sub-saturating light, but is relatively unaffected by OA under high light intensities.

Based on our flux measurements, we are able to provide an explanation for such differential OA sensitivities: In contrast to the low-light acclimated cells, where CO₂ uptake was strongly stimulated when being exposed to acidified conditions, CO₂ uptake in the high-light acclimated cells remained unaffected (Fig. 3b). Similar to the low-light acclimated cells, HCO₃⁻ uptake in the high-light acclimated cells was impaired under acidification (Fig. 3d). As a result, the overall Cᵢ uptake and consequently photosynthetic O₂ evolution were significantly decreased (Fig. 2b). The inability of high-light acclimated cells to increase CO₂ uptake may not only be the reason for the Cᵢ shortage under short-term acidification, but also explains why photosynthesis is often not stimulated after acclimation to OA (Raven & Crawfurd, 2012). However, a detrimental H⁺ effect on
photosynthesis in *E. huxleyi* has not yet been observed after acclimation, indicating that either the decrease in HCO$_3^-$ uptake is less pronounced, or the increase CO$_2$ uptake is more pronounced when cells are exposed to acidified conditions over an extended period of time.

The reduced capability of high-light acclimated cells to increase CO$_2$ uptake under acidification may derive from adjustments of their CO$_2$-concentrating mechanism (CCM) to the higher acclimation irradiance. *Emiliana huxleyi* was shown to increase HCO$_3^-$ uptake with increasing irradiance during flux measurements (Fig. 3d; 180 vs 700 PFD). This may indicate that high-light acclimated cells also used a higher fraction of HCO$_3^-$ under the conditions, at which they were cultured (Rost et al., 2006). Cells operating CCMs that are based predominantly on HCO$_3^-$ uptake need to reduce the diffusive losses and therefore downregulate their CO$_2$ permeability, for example by altering chloroplast morphology (Sukenik et al., 1987).

Recent studies on the combined effects of OA and light indicate that similar mechanisms, as here observed for *E. huxleyi*, also apply to other phytoplankton taxa. In diatoms, for example, growth was shown to increase significantly when cultured under OA and sub-saturating light, whereas these responses were reversed under high light (Gao et al., 2012). Besides light intensity, light fluctuations also have been shown to significantly modulate OA effects (Jin et al., 2013; Hoppe et al., 2015). Hoppe and coworkers, for example, observed that photosynthesis stayed constant under OA and constant light, but decreased under OA and dynamic light. According to our data, OA may generally lower the HCO$_3^-$ uptake capacity of phytoplankton. Although this is apparently not detrimental under low and stable light conditions, the impaired HCO$_3^-$ uptake seems to have severe consequences under high and dynamic light conditions. Under the latter conditions, the phytoplankton cells are dependent primarily on HCO$_3^-$ transport, because the high-Ci demand under high light and the varying Ci demand under dynamic light cannot be covered or adjusted fast enough by diffusive CO$_2$ uptake. Owing to the impairment of HCO$_3^-$ transporters, these cells are thus more prone to C$_i$ shortage at RubisCO under OA, even though external substrate concentrations are slightly elevated. When RubisCO becomes C$_i$-limited, the Calvin Cycle is a weaker electron sink, which can cause energetic overloads and higher costs associated with dissipation of energy and repair mechanisms (van de Poll et al., 2007; Gao et al., 2012; Jin et al., 2013; Hoppe et al., 2015). Thus, the high H$^+$-driven decrease in cellular HCO$_3^-$ uptake can explain why the energy transfer efficiency from photochemistry to biomass production is reduced under OA in combination with high or dynamic light conditions (Gao et al., 2012; Hoppe et al., 2015).

Haplots are insensitive to carbonate chemistry

The comparison of the two life-cycle stages of *E. huxleyi* revealed that their modes of C$_i$ acquisition strongly diverge. Photosynthetic and respiratory O$_2$ fluxes in the haploid stage did not respond to the short-term changes in carbonate chemistry (Table 2). Also CO$_2$ and HCO$_3^-$ uptake were not affected by carbonation or acidification. This agrees with the results of acclimation studies that often found no or few changes in POC production and other cellular processes under OA (Rokitta & Rost, 2012; Kottmeier et al., 2014). The fact that HCO$_3^-$ uptake was unaffected by external H$^+$ levels implies that the HCO$_3^-$ uptake mechanism of the haplont is different from the one of the diplont (Table 2). Indeed, there are transcriptomic datasets demonstrating that the two life-cycle stages express different isoforms of HCO$_3^-$ transporters of the SLC4 family (von Dassow et al., 2009; Mackinder et al., 2011; Rokitta et al., 2011). Also, the haplont was shown to express stage-specific subunits of a vacuolar H$^+$ ATPase and other stage-specific ion transporters, e.g. a Ca$^{2+}$/H$^+$ antiporters, which may further explain the differential sensitivity towards H$^+$ levels (von Dassow et al., 2009; Rokitta et al., 2011, 2012).

The consistently high HCO$_3^-$ usage of the haplont was not in line with the results of a $^{14}$C disequilibrium method, which estimated generally higher CO$_2$ contributions and a strong dependency on [CO$_2$]/pH (Kottmeier et al., 2014). This discrepancy may be attributed to the different key assumptions of the MIMS and/or the $^{14}$C disequilibrium methods. Regarding the MIMS method, we tested the consequences of potential offsets in key assumptions (e.g. variations in rate constants, PIC : POC, or photosynthetic quotient (PQ)) and found that typical uncertainties cannot explain the strong deviations between the methods. In contrast to the MIMS approach, the $^{14}$C disequilibrium technique does not yield actual CO$_2$ and HCO$_3^-$ uptake rates, but estimates the relative CO$_2$ uptake for photosynthesis (Lehman, 1971; Espie & Colman, 1986; Elzenga et al., 2000; Kottmeier et al., 2014). In this method, $f_{CO_2}$ is assessed based on the curvature of the cellular photosynthetic $^{14}$C incorporation during a transient isotopic $^{14}$CO$_2$ disequilibrium in the medium. In order to estimate $f_{CO_2}$, the $^{14}$C-incorporation is fitted with a model that is based on a number of parameters (Lehman, 1971; Espie & Colman, 1986). Some of these parameters, including kinetic constants, decay rates and the height of isotopic disequilibria remain error-afflicted and are currently being re-evaluated (S. Thoms et al. unpublished). Until these methodological discrepancies are better understood, the conflicting results for the haploid stage remain puzzling.

Impaired HCO$_3^-$ uptake under acidification may affect calcification

Although the strong negative H$^+$ effects on photosynthetic HCO$_3^-$ uptake have not explicitly been described before, negative H$^+$ effects on calcification are often discussed (Taylor et al., 2011; Fukuda et al., 2014; Bach et al., 2015; Cyronak et al., 2015). These inhibitory effects have often been attributed to changes in electrochemical gradients and the associated costs of H$^+$ removal (Mackinder et al., 2010; Raven, 2011; Suffrian et al., 2011; Taylor et al., 2011). In agreement with Fukuda et al., 2014, we here found strong evidence that acidification impairs the HCO$_3^-$ uptake. Assuming that high H$^+$ levels affect the transport of HCO$_3^-$ across the plasmalemma, the decreased uptake would not only influence photosynthesis, but also
calcification. Based on flux measurements of this study, we illustrated the presumed cellular Ci fluxes in response to typical OA scenarios under different light acclimations (Fig. 4).

As HCO$_3^-$ fluxes into POC and PIC are similar in magnitude, calcification may serve intrinsic pH regulation (Sikes et al., 1980; Price et al., 2008; Raven, 2011). More specifically, when HCO$_3^-$ is used for photosynthesis, one H$^+$ is consumed per fixed CO$_2$, and when HCO$_3^-$ is used for calcification, one H$^+$ is released per produced CaCO$_3$ (Fig. 4; Holtz et al., 2015a). The additional photosynthetic CO$_2$ uptake observed under acidification does not interfere with such a pH-homeostatic behaviour. Thus, independent of the external carbonate chemistry and light conditions, the need to exchange H$^+$ with the environment seems to be generally lower in the calcifying diplont of E. huxleyi (Fig. 4). This could provide the calcifying stage with an advantage over noncalcifying HCO$_3^-$ users (such as the haplont), which have to assure constant H$^+$ uptake to compensate for alkalinization during HCO$_3^-$-based photosynthesis (Raven, 1986, 2011). This advantage may add to the diplont’s success under bloom conditions, where seawater H$^+$ levels can become low.

**Conclusions**

In this study, we reveal a strong H$^+$-driven regulation of photosynthetic Ci fluxes of E. huxleyi that contradicts the commonly assumed ‘fertilizing effect’ of CO$_2$. At typical present-day conditions, HCO$_3^-$ was shown to be the major photosynthetic Ci source of both life-cycle stages. High H$^+$ levels were shown to rapidly inhibit the HCO$_3^-$ uptake and concomitantly to stimulate the CO$_2$ uptake. This H$^+$-dependent inhibition in HCO$_3^-$ uptake serves as a mechanistic explanation for the typical OA-dependent decline in calcification of coccolithophores and other marine calcifiers. Such an inhibition may be widespread among various phytoplankton taxa and also elucidates how the light-use efficiency can decrease when phytoplankton communities are grown under OA in combination with high or fluctuating light intensities. Future research should investigate whether similar
H⁺-dependent flux regulations are also evident when cells are acclimated to altered conditions.

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Author contributions

D.M.K., S.D.R. and B.R. planned and designed the research. D.M.K. performed experiments and analysed the data. S.D.R. and B.R. interpreted the data and wrote the manuscript.

References


Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Acclimation carbonate chemistry

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