



# Lower Salinity Leads to Improved Physiological Performance in the Coccolithophorid *Emiliana huxleyi*, Which Partly Ameliorates the Effects of Ocean Acidification

Jiekai Xu<sup>1</sup>, Jiazhen Sun<sup>1</sup>, John Beardall<sup>1,2</sup> and Kunshan Gao<sup>1,3\*</sup>

<sup>1</sup> State Key Laboratory of Marine Environmental Science, College of Ocean and Earth Sciences, Xiamen University, Xiamen, China, <sup>2</sup> School of Biological Sciences, Monash University, Clayton, VIC, Australia, <sup>3</sup> Co-Innovation Center of Jiangsu Marine Bio-industry Technology, Jiangsu Ocean University, Lianyungang, China

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### \*Correspondence:

Kunshan Gao  
ksgao@xmu.edu.cn

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While seawater acidification induced by elevated CO<sub>2</sub> is known to impact coccolithophores, the effects in combination with decreased salinity caused by sea ice melting and/or hydrological events have not been documented. Here we show the combined effects of seawater acidification and reduced salinity on growth, photosynthesis and calcification of *Emiliana huxleyi* grown at 2 CO<sub>2</sub> concentrations (low CO<sub>2</sub> LC:400 μatm; high CO<sub>2</sub> HC:1000 μatm) and 3 levels of salinity (25, 30, and 35‰). A decrease of salinity from 35 to 25‰ increased growth rate, cell size and photosynthetic performance under both LC and HC. Calcification rates were relatively insensitive to salinity though they were higher in the LC-grown compared to the HC-grown cells at 25‰ salinity, with insignificant differences under 30 and 35‰. Since salinity and OA treatments did not show interactive effects on calcification, changes in calcification:photosynthesis ratios are attributed to the elevated photosynthetic rates at lower salinities, with higher ratios of calcification to photosynthesis in the cells grown under 35‰ compared with those grown at 25‰. In contrast, photosynthetic carbon fixation increased almost linearly with decreasing salinity, regardless of the pCO<sub>2</sub> treatments. When subjected to short-term exposure to high light, the low-salinity-grown cells showed the highest photochemical effective quantum yield with the highest repair rate, though the HC treatment enhanced the PSII damage rate. Our results suggest that, irrespective of pCO<sub>2</sub>, at low salinity *Emiliana huxleyi* up-regulates its photosynthetic performance which, despite a relatively insensitive calcification response, may help it better adapt to future ocean global environmental changes, including ocean acidification, especially in the coastal areas of high latitudes.

**Keywords:** calcification, coccolithophore, CO<sub>2</sub>, *Emiliana huxleyi*, ocean acidification, photosynthesis, salinity

## INTRODUCTION

Since the Industrial Revolution, atmospheric  $p\text{CO}_2$  levels have increased by approximately 40%, mainly due to anthropogenic emissions of  $\text{CO}_2$  (Howes et al., 2015), and the oceans have absorbed about one third of the  $\text{CO}_2$  attributable to fossil fuel burning (Sabine et al., 2004), leading to increases in the concentration of  $p\text{CO}_2$ ,  $\text{HCO}_3^-$  and  $\text{H}^+$ , along with a decrease in the concentration of  $\text{CO}_3^{2-}$  and the saturation state of calcium carbonate, a process known as Ocean Acidification (OA). The pH of surface waters of the global ocean has already decreased by 0.1 units and will be further decreased by another 0.4 units by the end of this century under a “business-as-usual” scenario (Gattuso et al., 2015).

At the same time, the planet is also subject to global warming, another critical issue which has an important influence on organisms, with unforeseen consequences for marine biogeochemical cycling and ecosystem functioning (Taucher and Oschlies, 2011). It is predicted that the global mean temperature will increase by 2–5°C by 2100 (Gattuso et al., 2015). With warmer air and increasing ocean temperature, sea ice coverage has been declining, leading to a decrease in salinity, especially in coastal areas at high latitudes (Dickson et al., 2002; Ishii et al., 2006; Massom and Stammerjohn, 2010). For instance, Blindheim et al. (2000) and Albretsen et al. (2011) have reported a sharp decline in salinity in coastal areas of the Norwegian Sea during the last few decades, which in some locations has been more than 0.5‰ in the last 10 years. On the other hand, rainfall or hydrological changes associated with climate change can also alter surface seawater salinity. Changes in salinity in turn influence the solubility of  $\text{CO}_2$  in water and the equilibria between the different DIC species in the carbonate system, with decreased salinity resulting in small increases in  $\text{CO}_2$  solubility but with decreases in bicarbonate and carbonate concentrations (Zeebe and Wolf-Gladrow, 2001). As projected climate change processes are thought to amplify the effects of OA in the oceans of high latitude, organisms in the waters at high latitude are thought to be more vulnerable to rapid chemical changes (Chierici and Fransson, 2009; Qi et al., 2017). It is therefore of general interest to explore how marine photosynthetic organisms respond to changes in surface ocean carbonate chemistry and salinity that are potentially altered by both decreased salinity and rising  $\text{CO}_2$  concentration.

Coccolithophores, one of the most important calcifying primary producer groups, play a key role in the global carbon cycle, contributing about 10% to total organic carbon fixation (Poulton et al., 2007) and 50% to  $\text{CaCO}_3$  found in pelagic sediments (Broecker and Clark, 2009). The globally most abundant coccolithophore species is *Emiliania huxleyi*, which forms extensive blooms (Moore et al., 2012). With respect to *E. huxleyi*'s distinct sensitivity to OA, there have already been a number of studies that have documented its morphological, physiological and molecular responses to OA (Paasche, 2001; Raven and Crawford, 2012). Riebesell et al. (2000) reported negative effects of OA on morphology, calcification and growth on *E. huxleyi*, while Iglesias-Rodriguez et al. (2008) reported higher particulate inorganic and organic carbon (PIC, POC) per

cell under elevated  $p\text{CO}_2$ . Nevertheless, both studies showed decreased calcification rates under elevated  $p\text{CO}_2$  (Riebesell et al., 2008). Hoppe et al. (2011) showed that the PIC production of two *E. huxleyi* strains was negatively affected by higher  $p\text{CO}_2$ , although marked differences of responses to OA did exist among strains (Langer et al., 2009). Other environmental drivers are known to interact with OA to affect *E. huxleyi* (Gao et al., 2012; Boyd et al., 2018). For instance, exposure to UV radiation or high levels of photosynthetically active radiation (PAR) exacerbated impacts of OA on calcification (Feng et al., 2008; Gao et al., 2009). On the other hand, a recent study demonstrated that high levels of sunlight counteracted the negative effects of OA on calcification of *E. huxleyi* when the cells were grown under fluctuating incident solar radiation (Jin et al., 2017). Over 150–1000 generations, lower PIC:POC ratios were still observed in coccolithophorid species under OA (Müller et al., 2010; Lohbeck et al., 2012; Tong et al., 2018). A decreased PIC:POC ratio in another coccolithophorid, *Gephyrocapsa oceanica*, adapted to OA over 1000 generations persisted when cells were transferred back to the low  $p\text{CO}_2$  treatment, indicating an adaptive evolution (Tong et al., 2018). In brief, it is generally accepted that OA decreases calcification, and discrepancies exist when OA impacts on *E. huxleyi* in combination with other factors or under multiple drivers.

Changes in salinity are known to affect the morphology of coccolithophores (Bollmann and Herrle, 2007). While some *E. huxleyi* strains can survive at a salinity as low as 15‰ (Brand, 1984), its calcification is strongly depressed under such conditions, with malformed coccoliths (Saruwatari et al., 2016). Observations from sediment cores also showed that coccolith morphology was dependent on salinity (Fielding et al., 2009), and salinity can affect the hydrogen isotopic composition of long chain alkenones synthesized by *E. huxleyi* (Schouten et al., 2006). Reduction of salinity led to a smaller cellular volume in *E. huxleyi* due to reduced thickness of coccoliths (Green et al., 1998; Saruwatari et al., 2016). On the other hand, decreased salinity may interact with lowered pH to affect *E. huxleyi*, though it is known as a euryhaline species (Birkenes and Braarud, 1952; Paasche et al., 1996). Nevertheless, to the best of our knowledge, the combined effects of reduced salinity and lowered pH on *E. huxleyi* have not yet been documented. Given the effects of salinity on the carbonate system we therefore hypothesized that, in addition to the well documented consequences of changes in salinity for osmoregulation and metabolism, salinity and OA, and their consequences for concentrations of components of the carbonate system ( $\text{CO}_2$ , bicarbonate and carbonate), may interact in modulating the photosynthetic physiology, calcification rates and growth of *E. huxleyi*. Specifically, increased  $\text{CO}_2$  solubility might improve photosynthesis at low salinity, while the lower bicarbonate concentrations under such conditions would diminish cells' capacity for calcification. At the same time, decreased pH (ocean acidification) causes higher dissolved  $\text{CO}_2$ , and slightly higher bicarbonate concentrations and thus might be expected to interact with salinity to influence photosynthetic physiology and growth. In this study, we show that *Emiliania huxleyi* responds to hyposalinity by increasing photosynthetic performance and

growth, even when grown under OA conditions (though both parameters were decreased at high  $p\text{CO}_2$  at all salinities), despite a lack of effect on calcification and lower calcification to photosynthesis ratios under lower salinity conditions.

## MATERIALS AND METHODS

### Cultures and Experimental Setup

*Emiliana huxleyi* PML B92/11 was originally obtained from coastal waters off Bergen, Norway. This strain calcifies, but after having been maintained in laboratory culture for about 10 years in sterilized seawater enriched with Aquil medium (Sunda et al., 2005), its calcification capacity has decreased (Jin et al., 2017). No Si was added to the seawater but the nitrate level was initially adjusted to  $100 \mu\text{mol/L}$  and the phosphate concentration to  $10 \mu\text{mol/L}$ . Monospecific cultures (without aeration) were maintained at  $15^\circ\text{C}$  and illuminated by cool white fluorescent light at an intensity of  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , under a 12:12 h light and dark cycle.

Before inoculation, the culture medium was equilibrated with filtered ( $0.22 \mu\text{m}$ , Whatman) ambient air (from outdoors) of low ( $400 \mu\text{atm}$ ) or elevated ( $1000 \mu\text{atm}$ )  $\text{CO}_2$  concentrations prior to inoculation. The elevated  $\text{CO}_2$  was achieved by using a  $\text{CO}_2$  enriching device (CE100B, Wuhan Ruihua Instrument & Equipment Ltd., China) which gives rise to less than 5% variation of the controlled  $\text{CO}_2$  level. We set three levels of salinity (25, 30, 35‰): 35‰ is the salinity of our artificial seawater (Harrison and Berges, 2005) and 30‰ is close to the salinity of the strain's original environment (under the influence of the Norwegian coastal current) (Mork et al., 1981). The lowest level served as an extreme that is within the range of values occurring frequently in coastal and estuarine environments (Brand, 1984; Li et al., 2011). Reduced salinity was obtained by diluting prepared artificial seawater with Milli-Q water before sterilization, nutrient addition and aeration. Prior to inoculation, samples were collected for carbonate chemistry measurements (see the following section) and the seawater with the target  $p\text{CO}_2$  was divided carefully into three 500 ml polycarbonate bottles before inoculation. There were thus triplicate independent cultures for each treatment where the volume of each was 480 ml.

We used low biomass density batch cultures in sealed polycarbonate bottles (see below), shaken manually every day (LaRoche et al., 2010). Before inoculation, exponential growing cells had been pre-acclimated to the experimental conditions for at least 8 generations (5–6 days depending on growth condition). *E. huxleyi* was inoculated to about  $400 \text{ cells ml}^{-1}$ . Experimental cultures were then run for another 6–8 generations in the mid-exponential phase with gentle shaking twice a day. Cell concentrations never exceeded  $60,000 \text{ cells ml}^{-1}$ . Within the ranges of cell concentrations and carbonate chemistry parameters, we ensured that the cultures were still in the exponential phase (Zondervan et al., 2002) when experimental measurements were carried out.

Immediately before collecting the samples for measurement of experimental parameters, we took samples again for carbonate chemistry measurements to check that the carbonate system had

not been significantly perturbed despite culture growth. Samples for measurements of physiological parameters were all taken about 2.5 h after the onset of the light period on the last day of incubation, when cells had been growing under each treatment for about 14 generations.

### Carbonate Chemistry Sampling and Measurements

pH was measured by a pH meter (Orion 2 STAR, Thermo Fisher Scientific) calibrated with standard National Bureau of Standards (NBS) buffers. Samples (60 ml) for total alkalinity (TA) measurements were filtered ( $\sim 0.7 \mu\text{m}$ ), poisoned with a saturated  $\text{HgCl}_2$  solution (0.5‰ final concentration), and stored at  $4^\circ\text{C}$ . TA was measured in triplicate (each of 3 replicate cultures) and determined by Gran acidimetric titration with a TA analyzer (AS-Alk1+, Apollo SciTech). Certified reference materials (A. Dickson, LaJolla, CA, United States) from the Scripps Institution of Oceanography were used to assure the accuracy of the TA measurement. TA and pH data were applied to CO2SYS software (Lewis et al., 1998) to calculate other carbonate chemistry parameters (Table 1).

### Specific Growth Rate and Mean Cell Size Determination

Cell concentration and mean cell size were measured, using 5 ml samples, by a Coulter Particle Count and Size Analyzer (Z2, Beckman Coulter). Specific growth rate was calculated according to the equation:  $\mu = (\ln N_1 - \ln N_0)/(t_1 - t_0)$ , in which  $N_1$  and  $N_0$  represent cell concentrations at  $t_1$  and  $t_0$ , where  $t_0$  represents the beginning and  $t_1$  the end day of the cultures. The period of ( $t_1 - t_0$ ) fell within the exponential growth phase as described above.

### Chlorophyll and Carotenoid Contents

Cells (15 ml) for determination of pigment content were filtered onto GF/F filters (25 mm, Whatman) and then extracted overnight in absolute methanol at  $4^\circ\text{C}$  in darkness (Li et al., 2017). After centrifugation (5000 g for 10 min), the absorption values of the sample supernatants were analyzed by a UV-VIS spectrophotometer (DU800, Beckman Coulter). The concentrations of chlorophylls *a* and *c* were calculated according to Ritchie (2006):

$$\text{Chl } a \text{ (}\mu\text{gml}^{-1}\text{)} = 13.2654 * (A_{665} - A_{750}) - 2.6839 * (A_{632} - A_{750})$$

$$\text{Chl } c \text{ (}\mu\text{gml}^{-1}\text{)} = -6.0138 * (A_{665} - A_{750}) + 28.8191 * (A_{632} - A_{750})$$

Carotenoid concentration was determined by the equation given by Strickland and Parsons (1972):

$$\text{Carotenoid (}\mu\text{gml}^{-1}\text{)} = 7.6 * ((A_{480} - A_{750}) - 1.49 * (A_{510} - A_{750}))$$

where  $A_x$  indicates the absorbance at a wavelength  $x$ . The pigment content per cell was calculated by taking the dilution factor and cell concentration into account.

**TABLE 1** | Carbonate chemistry parameters in the cultures of *E. huxleyi* before (B) inoculation (already equilibrated with the ambient or elevated CO<sub>2</sub> levels) and after (A) about 8 generations prior to determination of physiological parameters.

Treatment	PH <sub>NBS</sub>	TA (μmol kg <sup>-1</sup> )	DIC (μmol kg <sup>-1</sup> )	HCO <sub>3</sub> <sup>-</sup> (μmol kg <sup>-1</sup> )	CO <sub>3</sub> <sup>2-</sup> (μmol kg <sup>-1</sup> )	CO <sub>2</sub> (μmol kg <sup>-1</sup> )	pCO <sub>2</sub> (ppm)
25‰							
LC(B)	8.01 ± 0.01 <sup>ab</sup>	1480.25 ± 8.64 <sup>c</sup>	1394.12 ± 10.06 <sup>f</sup>	1318.92 ± 10.15 <sup>g</sup>	58.22 ± 0.50 <sup>c</sup>	16.97 ± 0.41 <sup>c</sup>	430.16 ± 10.31 <sup>c</sup>
LC(A)	7.80 ± 0.02 <sup>c</sup>	1431.04 ± 87.94 <sup>c</sup>	1388.55 ± 83.43 <sup>f</sup>	1324.60 ± 79.20 <sup>g</sup>	36.87 ± 3.87 <sup>ef</sup>	27.07 ± 0.38 <sup>b</sup>	685.92 ± 9.69 <sup>b</sup>
HC(B)	7.61 ± 0.01 <sup>e</sup>	1510.52 ± 23.74 <sup>c</sup>	1505.25 ± 25.47 <sup>ef</sup>	1434.14 ± 23.97 <sup>ef</sup>	25.79 ± 0.01 <sup>f</sup>	45.32 ± 1.50 <sup>a</sup>	1148.25 ± 37.88 <sup>a</sup>
HC(A)	7.64 ± 0.02 <sup>de</sup>	1487.42 ± 15.34 <sup>c</sup>	1476.91 ± 15.73 <sup>ef</sup>	1408.05 ± 14.91 <sup>ef</sup>	26.83 ± 1.08 <sup>f</sup>	42.02 ± 1.77 <sup>a</sup>	1064.69 ± 44.92 <sup>a</sup>
30‰							
LC(B)	8.07 ± 0.01 <sup>a</sup>	1735.08 ± 8.33 <sup>b</sup>	1600.10 ± 10.23 <sup>de</sup>	1496.45 ± 10.68 <sup>de</sup>	88.29 ± 0.81 <sup>b</sup>	15.36 ± 0.36 <sup>c</sup>	400.29 ± 9.37 <sup>c</sup>
LC(A)	7.96 ± 0.01 <sup>b</sup>	1704.14 ± 107.22 <sup>b</sup>	1603.63 ± 101.65 <sup>de</sup>	1514.36 ± 95.09 <sup>de</sup>	69.17 ± 6.05 <sup>c</sup>	20.10 ± 1.09 <sup>c</sup>	523.61 ± 28.48 <sup>bc</sup>
HC(B)	7.68 ± 0.01 <sup>de</sup>	1787.40 ± 19.06 <sup>b</sup>	1756.61 ± 20.79 <sup>cd</sup>	1674.18 ± 19.78 <sup>c</sup>	40.24 ± 0.18 <sup>d<sup>ef</sup></sup>	42.19 ± 1.18 <sup>a</sup>	1099.34 ± 30.89 <sup>a</sup>
HC(A)	7.65 ± 0.02 <sup>d</sup>	1743.80 ± 21.78 <sup>b</sup>	1722.02 ± 27.18 <sup>cd</sup>	1640.67 ± 25.44 <sup>cd</sup>	36.12 ± 1.58 <sup>f</sup>	45.23 ± 3.28 <sup>a</sup>	1178.52 ± 85.43 <sup>a</sup>
35‰							
LC(B)	8.09 ± 0.01 <sup>a</sup>	1977.95 ± 0.64 <sup>a</sup>	1802.51 ± 3.42 <sup>bc</sup>	1671.57 ± 4.67 <sup>c</sup>	115.15 ± 1.55 <sup>a</sup>	15.78 ± 0.30 <sup>c</sup>	422.79 ± 8.06 <sup>c</sup>
LC(A)	8.00 ± 0.07 <sup>ab</sup>	1969.47 ± 7.73 <sup>a</sup>	1823.53 ± 31.61 <sup>abc</sup>	1705.43 ± 41.23 <sup>bc</sup>	98.63 ± 12.99 <sup>ab</sup>	19.48 ± 3.34 <sup>c</sup>	521.79 ± 89.43 <sup>bc</sup>
HC(B)	7.71 ± 0.01 <sup>de</sup>	2019.13 ± 0.66 <sup>a</sup>	1967.89 ± 2.67 <sup>a</sup>	1871.75 ± 2.72 <sup>a</sup>	53.75 ± 0.79 <sup>cde</sup>	42.39 ± 0.75 <sup>a</sup>	1135.63 ± 20.14 <sup>a</sup>
HC(A)	7.72 ± 0.01 <sup>cd</sup>	2002.71 ± 3.20 <sup>a</sup>	1946.33 ± 7.53 <sup>ab</sup>	1850.68 ± 7.69 <sup>ab</sup>	55.45 ± 1.73 <sup>cd</sup>	40.19 ± 1.57 <sup>a</sup>	1076.90 ± 42.09 <sup>a</sup>

Values are means ± SD of triplicate cultures. Different superscripted letters indicate significant differences ( $p < 0.05$ , Tukey-test). Between different treatment and timing.

## Chlorophyll a Fluorescence

The photochemical parameters were determined using a Multiple Excitation Wavelengths Chlorophyll Fluorescence Analyzer (Multi-color-PAM, Walz). Maximum and effective photochemical quantum yields were determined according to the equations of Genty et al. (1989): maximum photochemical quantum yield ( $F_v/F_m$ ) =  $(F_m - F_0)/F_m$ , for dark-adapted (10 min) samples; effective photochemical quantum yield  $\Phi_{PSII}$  ( $F'_v/F'_m$ ) =  $(F'_m - F_t)/F'_m$  for light-adapted samples, where  $F_m$  and  $F'_m$  indicate maximum chlorophyll fluorescence of dark and growth-light-adapted samples, respectively,  $F_0$  is the minimum chlorophyll fluorescence of dark-treated cells; and  $F_t$  is the steady-state chlorophyll fluorescence of light exposed samples.  $\Phi_{PSII}$  was measured under an actinic light intensity ( $\sim 240 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) similar to the culture light level. The saturation pulse was set at  $5000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and lasted for 0.8 s.

Another experiment was carried out on the last day of incubations, investigating the response of cells under each treatment to high light exposure. Samples were placed in 35 ml quartz tubes wrapped with Ultraphan film 395 (UV Opak, Digefra), receiving irradiances above 395 nm (PAR). The transmission details of this cut-off foil and quartz tubes are available elsewhere (Gao et al., 2007). The tubes for fluorescence measurements were placed under a solar simulator (Sol 1200W; Dr. Hönle, Martinsried, Germany). The cells were exposed to PAR of  $800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 48 min at 15°C (growth temperature) while maintaining the tubes in a circulating water bath (CTP-3000, Eyela) for temperature control. During the exposures, measurements of fluorescence parameters were carried out (see above). Aliquots of 2.5 ml of sample from each tube (total of 18 tubes, that is triplicates of each treatment) were taken every 3–10 min during exposure, and immediately measured (without any dark adaptation) to get the instant maximum fluorescence  $F'_m$  and the steady state fluorescence  $F_t$

of the light adapted cells, and  $\Phi_{PSII}$  was calculated as described above. The rates of high light-induced damage to PSII ( $k$ ,  $\text{min}^{-1}$ ) and the corresponding repair rates ( $r$ ,  $\text{min}^{-1}$ ) were estimated according to Beardall et al. (2014), applying the Kok model that assumes simultaneous operation of the damage and recovery during the photoinhibitory exposures, and calculated as follows:

$$P_n/P_0 = r/(r+k) + k/(r+k) * \exp(-(r+k) * t)$$

where  $P_n$  and  $P_0$  represent effective quantum yield values at time  $t$  (minutes) and time zero, respectively.

## Determination of Photosynthetic and Calcification Rates

After the exponentially grown cells had been acclimated to 6–8 generations, the cells were collected in the middle of the photoperiod, dispensed into borosilicate bottles (20 ml) and inoculated with 5  $\mu\text{Ci}$  (0.185 MBq) of labeled sodium bicarbonate (Amersham). After 2 h of incubation under the same environment as the cultures, samples were immediately filtered under dim light onto Whatman GF/F glass fiber filters (25 mm), rinsed with unlabeled medium, placed in 20 mL scintillation vials, and then dried (60°C, 5 h) before counting in a liquid scintillation counter (Beckman, LS6500) with 5 ml scintillation cocktail (Perkin Elmer). In parallel, another filter with the cells was fumed with HCl overnight to expel non-fixed <sup>14</sup>C for measurement of photosynthetic <sup>14</sup>C fixation into organic (acid stable) material. Calculation of rates of photosynthesis took into account the different specific activities resulting from the changes in [DIC] with salinity and pCO<sub>2</sub> as shown in Table 1. The rate of calcification of *E. huxleyi* was estimated as the difference between the total (inorganic and organic) and the photosynthetic carbon fixation (Paasche, 1963; Gao et al., 2009). Two samples for calcification (30‰ HC, 35‰ LC) were lost during measurements, so are not reported in the results.

## Statistical Analyses

Before performing parametric tests, data were tested for homogeneity of variance (Levene test) and normality (Shapiro-Wilk test). Data were analyzed by Two-way ANOVA in SPSS software, with  $p\text{CO}_2$  (two levels), salinity (three levels) and the interaction classed as factors in the model. When  $p < 0.05$ , a Tukey test was conducted as a *post hoc* (One-way ANOVA) test to assess significant differences among the three levels of salinity. Since two  $p\text{CO}_2$  levels were applied, an independent-samples *t*-test was employed to determine differences between the  $p\text{CO}_2$  levels. Data are reported as means  $\pm$  SD. A 95% confidence level was used in all analyses. Where we have discussed the percentage change in parameters we used the formula

$$\text{Percentage change} = \frac{(V_{\text{compared}} - V_{\text{comparison}})}{V_{\text{comparison}}} \times 100$$

where *V* represents the value of the physiological metric (such as growth rate).

## RESULTS

Most parameters, as outlined below, showed statistically significant effects of salinity and  $p\text{CO}_2$  alone. With the exceptions of growth rate, cell diameter, quantum yield of PSII ( $F_v/F_m$ ) and calcification all other parameters showed significant interaction between  $p\text{CO}_2$  (OA) and salinity (Table 2).

### Specific Growth Rate and Cell Size

No interactive effects were found, but individual effects of salinity and  $p\text{CO}_2$  were observed on specific growth rate, and the contribution of the  $p\text{CO}_2$  effect was greatest according to the magnitude of  $\eta^2$  (Table 2, Two-way ANOVA,  $p = 0.757$ ,  $p = 0.001$ ,  $p < 0.001$ , respectively). Under LC, growth rate increased significantly with decreasing salinity ( $R^2 = 0.88$ , Figure 1). There were significant differences in growth rate between cultures at 25 and 35‰, and significant differences were also found between 30 and 35‰ (Tukey-test,  $p = 0.009$ ,  $p = 0.022$ , respectively), while no significant difference was found in growth rate between cultures at 25 and 30‰ under both LC and HC treatments (Figure 1A, Tukey-test,  $p = 0.695$ ,  $p = 0.995$ ). Under LC, compared to 35‰, treatments with salinity levels of 25 or 30‰ enhanced growth rate by 10 and 8%, respectively. Under HC, decreases in salinity had a less marked effect on growth rate though salinities of 25 and 30‰ enhanced growth rate by 9 and 10% (Tukey-test,  $p = 0.150$ ,  $p = 0.103$ , respectively) compared to rates at 35‰. For all salinity treatments, OA treatment decreased the growth rate by 19, 16, and 18% (*t*-test,  $p = 0.001$ – $0.02$ ) compared to that of LC-grown cells under 25, 30, and 35‰, respectively.

Cell size (diameter) ranged from 4.20 to 4.88  $\mu\text{m}$ . Mean cell sizes were largest in the 25‰ treatment and smallest at 30‰ under both LC and HC (Figure 1B). OA decreased cell size by 2, 4, and 5% (*t*-test,  $p = 0.000$ – $0.005$ ) compared to that of LC-grown cells under 25, 30, and 35‰, respectively. For cell size, there was no interaction between salinity and  $p\text{CO}_2$ , and the salinity

**TABLE 2** | Summary of Two-way ANOVA analyses for interactive effects of  $p\text{CO}_2$  and salinity on growth, photosynthesis, calcification, pigments, and quantum yield in *E. huxleyi*.

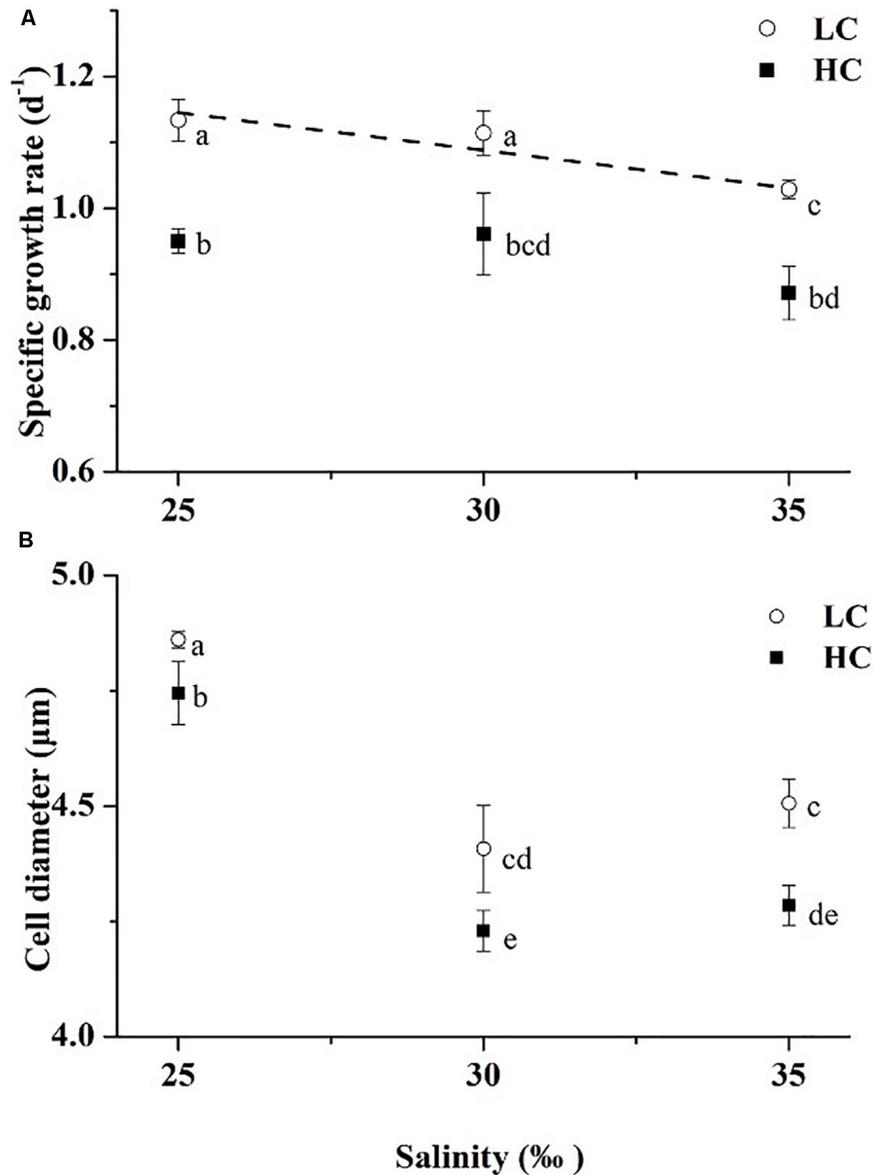
Variable, factors	df	Mean square	F	<i>p</i>	$\eta^2$
<b>Growth</b>					
$p\text{CO}_2$	1	0.122	89.482	<b>&lt;0.001</b>	<b>0.882</b>
Salinity	2	0.016	11.878	<b>0.001</b>	0.664
$p\text{CO}_2 \times \text{Salinity}$	2	0.000	0.285	0.757	0.045
<b>Cell diameter</b>					
$p\text{CO}_2$	1	0.122	34.520	<b>&lt;0.001</b>	0.758
Salinity	2	0.384	108.606	<b>0.001</b>	<b>0.952</b>
$p\text{CO}_2 \times \text{Salinity}$	2	0.004	1.181	0.343	0.177
<b>Chl a</b>					
$p\text{CO}_2$	1	0.002	48.006	<b>&lt;0.001</b>	0.800
Salinity	2	0.002	42.433	<b>&lt;0.001</b>	0.876
$p\text{CO}_2 \times \text{Salinity}$	2	0.002	55.102	<b>&lt;0.001</b>	<b>0.902</b>
<b>Chl c</b>					
$p\text{CO}_2$	1	<0.001	6.355	<b>0.027</b>	0.346
Salinity	2	<0.001	1.893	0.193	0.240
$p\text{CO}_2 \times \text{Salinity}$	2	0.001	69.331	<b>&lt;0.001</b>	<b>0.920</b>
<b>Fv/Fm</b>					
$p\text{CO}_2$	1	<0.001	0.137	0.718	0.011
Salinity	2	<0.001	0.960	0.410	0.138
$p\text{CO}_2 \times \text{Salinity}$	2	<0.001	1.063	0.376	0.150
<b>Photosynthesis</b>					
$p\text{CO}_2$	1	0.831	52.032	<b>&lt;0.001</b>	0.813
Salinity	2	2.050	128.262	<b>&lt;0.001</b>	<b>0.955</b>
$p\text{CO}_2 \times \text{Salinity}$	2	0.068	4.244	<b>0.040</b>	0.414
<b>Calcification</b>					
$p\text{CO}_2$	1	0.002	7.977	<b>0.018</b>	0.444
Salinity	2	0.001	4.138	<b>0.049</b>	<b>0.453</b>
$p\text{CO}_2 \times \text{Salinity}$	2	0.001	3.621	0.066	0.420
<b>C/P</b>					
$p\text{CO}_2$	1	0.022	5.028	<b>0.049</b>	0.335
Salinity	2	0.162	37.050	<b>&lt;0.001</b>	<b>0.881</b>
$p\text{CO}_2 \times \text{Salinity}$	2	0.049	11.286	<b>0.003</b>	0.693

The  $\eta^2$  was used as a measure of effect size. The symbol “x” indicates the interactions between factors. Bolded values emphasize that  $p < 0.05$  and the greatest  $\eta^2$ .

contributed the greatest effect according to the magnitude of  $\eta^2$  (Table 2, Two-way ANOVA,  $p = 0.343$ ,  $p = 0.001$ , respectively).

### Pigment Content

Significant interaction effects and individual effects of salinity and  $p\text{CO}_2$  on chlorophyll *a* content of cells were observed. The effects that contributed to the variability of chlorophyll *a* (in order of  $\eta^2$ ) were:  $p\text{CO}_2 \times \text{salinity} > \text{salinity} > p\text{CO}_2$  (Table 2, Two-way ANOVA,  $p < 0.001$ ). Growth at 25‰ increased chlorophyll *a* content of the cells by 72% (Tukey-test,  $p < 0.001$ ) compared to cells grown at 30 and 180% higher (Tukey-test,  $p < 0.001$ ) than those at 35‰ under HC (Figure 2A). Under LC, there were no significant differences between values in cells at 25 and 35‰ (Tukey-test,  $p = 0.914$ ), 30 and 35‰ (Tukey-test,  $p = 0.119$ ) while growth at 30‰ increased the chlorophyll *a* content of cells by 19% (Tukey-test,  $p = 0.071$ ) relative to 25‰. In the 25‰



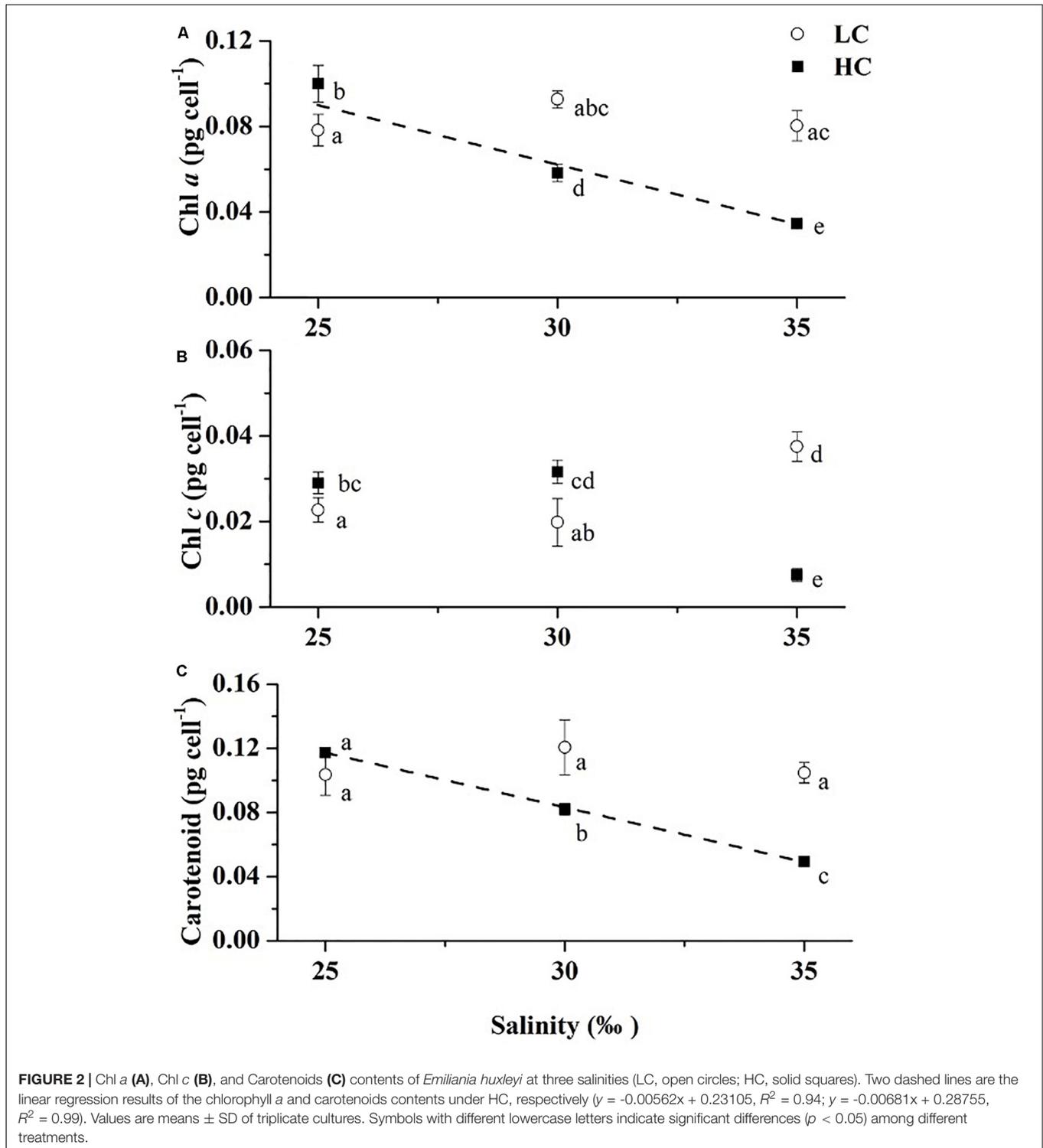
**FIGURE 1** | Specific growth rates (A) and cell diameters (B) of *Emiliana huxleyi* at three salinities (LC, open circles; HC, solid squares). The dashed line is the linear regression result of the growth rates under LC ( $y = -0.01142x + 1.43055$ ,  $R^2 = 0.88$ ). Lines in this and subsequent figures are only presented in cases where there were significant linear trends of parameters with salinity. Values are means  $\pm$  SD of triplicate cultures. Symbols with different lowercase letters indicate significant differences ( $p < 0.05$ ) among different treatments.

treatment, HC increased the chlorophyll *a* content of cells by 28% (*t*-test,  $p = 0.040$ ) relative to that of LC cells. However, chlorophyll *a* content was higher in the LC treatment than HC under both 30 and 35‰. LC increased chlorophyll *a* content by 60% (*t*-test,  $p < 0.001$ ) compared to HC under 30‰ and was 132% higher (*t*-test,  $p < 0.001$ ) than in the HC treatment under 35‰.

The trend of carotenoid content of cells was same as for chlorophyll *a* (Figure 2C) in that the value was higher in the LC treatment than at HC under 30 and 35‰, although there was no significant difference between LC and HC under 25‰ (*t*-test,

$p = 0.144$ ). There were also no significant differences among the three salinity treatments under LC (Tukey-test,  $p = 0.159$ – $0.920$ ). Under HC, the carotenoid content increased at the low salinity, by 43% (Tukey-test,  $p < 0.001$ ) and by 137% (Tukey-test,  $p < 0.001$ ) in the 25‰ treatment relative to 30 and 35‰, respectively.

Partial pressure of CO<sub>2</sub> ( $pCO_2$ ) and the interaction with salinity significantly impacted chlorophyll *c*, and the contribution of the interaction effect was predominant according to the magnitude of  $\eta^2$  (Table 2, Two-way ANOVA,  $p = 0.027$ ,  $p < 0.001$ ). Though no significant impact of salinity was observed



on chlorophyll c, there were significant differences in chlorophyll c content between cells grown at 25 and 35‰, and between 30 and 35‰ under both LC (Tukey-test,  $p = 0.011$ ,  $p = 0.005$ ) and HC (Tukey-test,  $p < 0.001$ ,  $p < 0.001$ ), while no significant difference was found between cells grown at 25 and 30‰ under both  $pCO_2$  levels (Figure 2B, Tukey-test,  $p = 0.680$ ,  $p = 0.400$ ). Among the

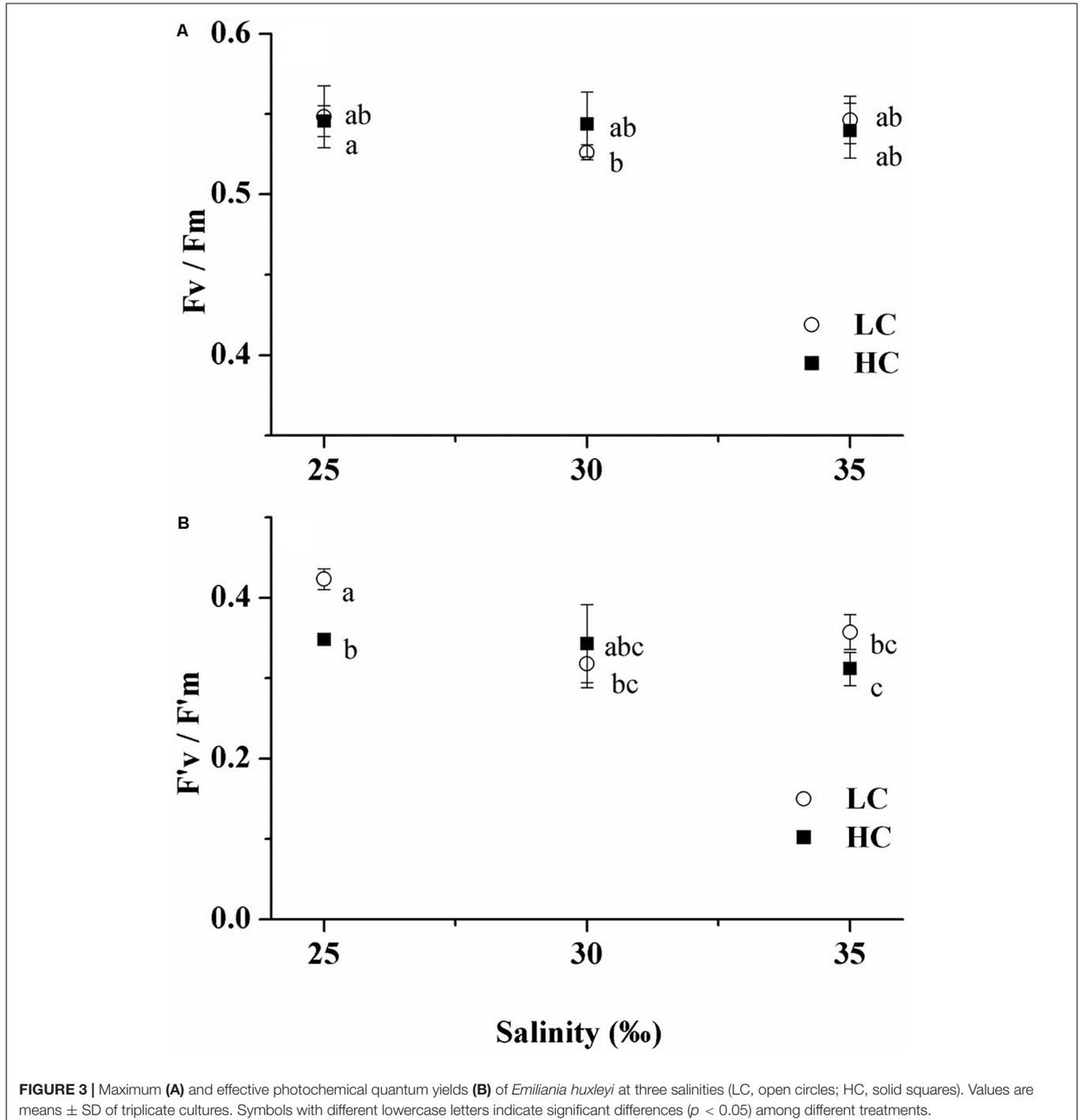
three salinity levels, only in the 35‰ treatment could a higher chlorophyll c content under LC than HC be observed.

### Chlorophyll a Fluorescence

The maximal photochemical quantum yields varied little among all treatments, ranging from  $0.53 \pm 0.01$  to  $0.55 \pm 0.02$

(Figure 3A). This suggests, *inter alia*, that nutrient levels were not depleted during the experimental period, as shown indirectly through the steady and relatively high maximal photochemical quantum yields, a parameter which is often applied to estimate nutrient status in the ocean (Falkowski et al., 2004). Elevated  $p\text{CO}_2$  scarcely influenced effective photochemical quantum yields under all salinity treatments (Figure 3B). The only exception was that  $\Phi_{\text{PSII}}$  in HC cells decreased by 17% ( $t$ -test,

$p < 0.001$ ) relative to that of LC cells under 25‰. Under LC, 25‰ grown cells showed stimulated values of  $\Phi_{\text{PSII}}$  by 33% (Tukey-test,  $p = 0.003$ ) and 18% (Tukey-test,  $p = 0.026$ ) compared to cells in 30 and 35‰, respectively. Under HC, though there was no significant difference among the three salinity treatments (Tukey-test,  $p = 0.368\text{--}0.975$ ), growth at 25‰ slightly increased  $\Phi_{\text{PSII}}$  by 12% relative to the 35‰ treatment (Figure 3B). Two-way ANOVA revealed no significant interaction between  $p\text{CO}_2$  and

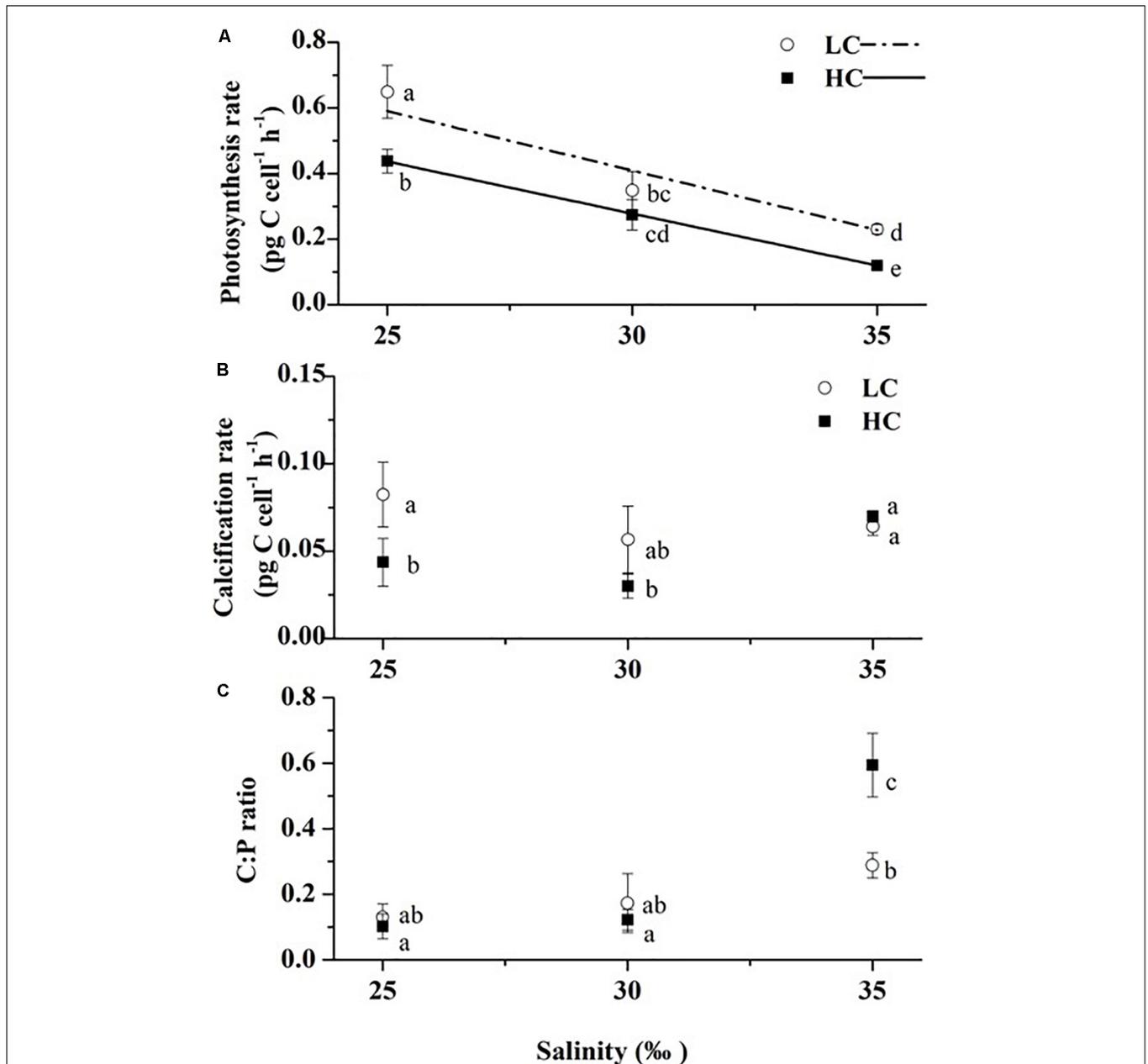


salinity on the maximal photochemical quantum yield (Table 2, Two-way ANOVA,  $p = 0.376$ ).

### Photosynthesis and Calcification

Photosynthetic carbon fixation increased with decreasing salinity (Figure 4A). Cells grown under 25‰ had a photosynthesis rate  $1.8 \times$  higher than in 35‰ under LC (Tukey-test,  $p < 0.001$ ) and  $2.7 \times$  higher (Tukey-test,  $p = 0.003$ ) than in 30‰ under HC. OA generally decreased photosynthetic carbon fixation with

the only exception being that no significant difference was found between LC and HC under 30‰ ( $t$ -test,  $p = 0.086$ ) although the mean value of the former was still 27% higher (Figure 4A). OA decreased photosynthetic carbon fixation by 33, 21, and 48% ( $t$ -test,  $p = 0.003$ – $0.086$ ) compared to that of LC-grown cells under 25, 30, and 35‰, respectively. Two-way ANOVA revealed a significant interaction between  $p\text{CO}_2$  and salinity, while the salinity effect was predominant (Table 2, Two-way ANOVA,  $p = 0.040$ ,  $p < 0.001$ ).



**FIGURE 4 |** Photosynthetic rates (A), calcification rates (B) at 6 h after the onset of light and calcification to photosynthesis ratio, C: P (C) of three salinities (LC, open circles; HC, solid squares). The dash-dotted line and solid line are the linear regression results of the photosynthetic rates under LC and HC, respectively ( $y = -0.03624x + 1.49612$ ,  $R^2 = 0.89$ ;  $y = -0.03173x + 1.22979$ ,  $R^2 = 0.99$ ). Values are means  $\pm$  SD of triplicate cultures. Symbols with different lowercase letters indicate significant differences ( $p < 0.05$ ) among different treatments.

The calcification rates ranged from  $0.03 \pm 0.01$  to  $0.08 \pm 0.02$   $\mu\text{g C cell}^{-1} \text{h}^{-1}$ . No significant differences were found among all salinity treatments under LC (Figure 4B, Tukey-test,  $p = 0.249$ – $0.886$ ). In contrast, under HC, growth at 35‰ stimulated the calcification rates of cells by 60% (Tukey-test,  $p = 0.042$ ) and by  $1.3 \times$  (Tukey-test,  $p = 0.013$ ) compared to values in cells grown at 25 and 30‰, respectively. An effect of OA was not found under the 35‰ treatment, while it decreased calcification rates by 47% under 25‰ ( $t$ -test,  $p = 0.044$ ) and 30‰, although there were no significant differences between LC and HC under the 30‰ treatment ( $t$ -test,  $p = 0.170$ ). No significant interaction effects of salinity and  $p\text{CO}_2$  on the calcification rates were found, though individual effects of salinity and  $p\text{CO}_2$  were significant (Table 2, Two-way ANOVA,  $p = 0.066$ ,  $p = 0.049$ ,  $p = 0.018$ , respectively). The contribution of the salinity effect was maximal according to the magnitude of  $\eta^2$  (Table 2).

Significant interaction effects and individual effects of salinity and  $p\text{CO}_2$  were found in the ratio of calcification (C) to photosynthesis (P) (Table 2, Two-way ANOVA,  $p < 0.05$ ). The effects that contributed to the variability of C:P (in order of  $\eta^2$ ) were: salinity > salinity  $\times$   $p\text{CO}_2$  >  $p\text{CO}_2$  (Table 2). Seawater acidification and high salinity acted synergistically to stimulate the C:P ratio, thus resulting in the highest value under the 35‰ HC treatment. Salinity seemed to play a more important role as it has the largest  $\eta^2$  and no significant differences between LC and HC were found, the only exception being under 35‰ where OA increased the ratio by 103% ( $t$ -test,  $p = 0.027$ ). With increasing salinity, the C:P ratio increased regardless of  $p\text{CO}_2$  levels. Under LC, the C:P value of cells grown under 35‰ was  $1.2 \times$  (Tukey-test,  $p = 0.095$ ) and by 67% higher (Tukey-test,  $p = 0.218$ ) than at 25 and 30‰, respectively, with the equivalent values being  $4.8 \times$  (Tukey-test,  $p = 0.001$ ) and  $3.9 \times$  (Tukey-test,  $p = 0.001$ ) higher under HC. However, given the minimal effects of salinity on calcification rates, increases in C:P are mostly attributable to lower photosynthetic rates at higher salinities.

## Photochemical Responses

When exposed to high light ( $800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), the effective photochemical quantum yield  $\Phi_{\text{PSII}}$  decreased significantly during the first 0–15 min of exposure in all salinity/ $p\text{CO}_2$  treatments (Figure 5); after this period,  $\Phi_{\text{PSII}}$  remained more or less constant. Regardless of  $p\text{CO}_2$  levels, the samples grown under 25‰ had a smaller decrease in  $\Phi_{\text{PSII}}$  than cells grown at the higher salinities.

This trend was especially clear under LC (Figure 5A). During the whole exposure process, the  $\Phi_{\text{PSII}}$  value of the cells under the 25‰ treatment was highest, with the lowest value reached being ca. 0.21 after 48 min exposure, which was  $4.4 \times$  (Tukey-test,  $p = 0.004$ ) and 80% (Tukey-test,  $p = 0.045$ ) higher than that of cells from 30 and 35‰ treatments, respectively. In contrast,  $\Phi_{\text{PSII}}$  of the 30‰ treatment was always lowest during the whole exposure process.

In the HC-grown cells,  $\Phi_{\text{PSII}}$  declined much faster compared to that in the LC-grown cells except that at some time points, the value of  $\Phi_{\text{PSII}}$  was higher in HC-grown cells under the 30‰ treatment (Figure 5B). No significant differences in  $\Phi_{\text{PSII}}$  among three salinity treatments were found during the whole exposure

(Figure 5B). Nonetheless,  $\Phi_{\text{PSII}}$  of the 25‰ treatment was still highest during the whole exposure process, with the lowest value reached being ca. 0.1.

After cells were exposed to short-term acute exposures to elevated levels of visible solar radiation, there was a significant difference in repair rates among the three salinity treatments under LC (Figure 6A). The highest  $r$  values were observed under the 25‰ treatment, which were  $4.3 \times$  (Tukey-test,  $p = 0.007$ ) and 72% higher (Tukey-test,  $p = 0.100$ ) than those of 30 and 35‰ treatments while no significant differences were observed under HC (Tukey-test,  $p = 0.175$ – $0.981$ ). No significant differences in damage rates,  $k$ , were observed under HC among three salinity treatments (Tukey-test,  $p = 0.135$ – $0.986$ ) (Figure 6B). While under LC,  $k$ -values were highest in the 30‰ treatment and reached about  $0.4 \text{ min}^{-1}$ , a value which was  $1.3 \times$  (Tukey-test,  $p = 0.001$ ) and 91% higher (Tukey-test,  $p = 0.003$ ) than those of the 25 and 35‰ treatments, respectively.

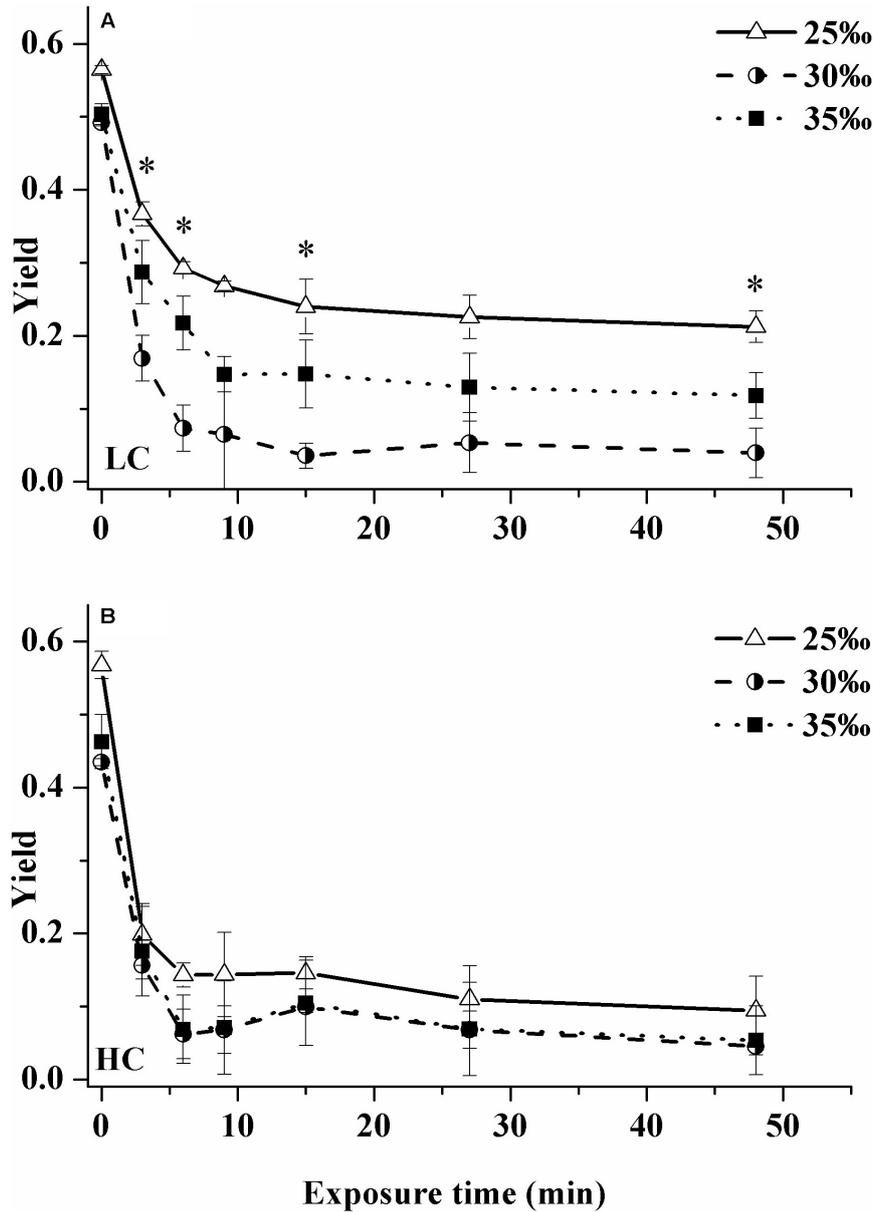
From the ratio of  $r:k$ , it is clear that under LC, cells grown at 25‰ showed improved ability to cope with high light stress than cells grown under higher salinities (Figure 6C). Changes in  $r:k$  across salinity were not marked under HC, attributable largely by the much higher damage rate ( $k$ ) at 25‰.

## DISCUSSION

Although the effects of OA and salinity have been reported extensively as separate factors influencing the performance of coccolithophores, to date the possible interaction between these environmental parameters has not been examined. In the present work, we found that salinity and  $p\text{CO}_2$  (OA), both individually and interactively, affected pigment content, photosynthesis and the ratio of photosynthesis to calcification, while growth and calcification only significantly responded to salinity or OA treatment alone (without interactions detected) (Table 2). It should be noted that interaction of salinity and  $p\text{CO}_2$  (OA) did not significantly affect calcification (Table 2).

Although we did not measure nutrient concentrations, but recognize that nutrient limitation can influence responses to OA (Boyd et al., 2018), we have no reason to suspect nutrient deficiency during our experiments. Nutrient deficiency results in a major impairment of photochemical efficiency. Declines in maximum photochemical efficiency  $F_v/F_m$  have been observed over a range of algal taxa for N-, P- or Fe-starved cells (e.g., Kolber et al., 1988; Greene et al., 1992, 1994; LaRoche et al., 1993; Berges et al., 1996; McKay et al., 1997).  $F_v/F_m$  has been shown to recover following nutrient re-addition following starvation and can be used as a measure of phytoplankton “health” for bioassays on natural populations (LaRoche et al., 1993; Geider and LaRoche, 1994; Boyd et al., 1999). Our data showed stable  $F_v/F_m$  values across the treatments and were comparable with values reported by Xu et al. (2016), suggesting that nutrient deprivation was not occurring during our experiments.

Calcification rate of *E. huxleyi* was little affected by salinity under LC but was reduced under HC at the 2 lower salinities tested here. At the highest salinity,  $p\text{CO}_2$  had no effect on calcification rate. However, photosynthetic carbon fixation

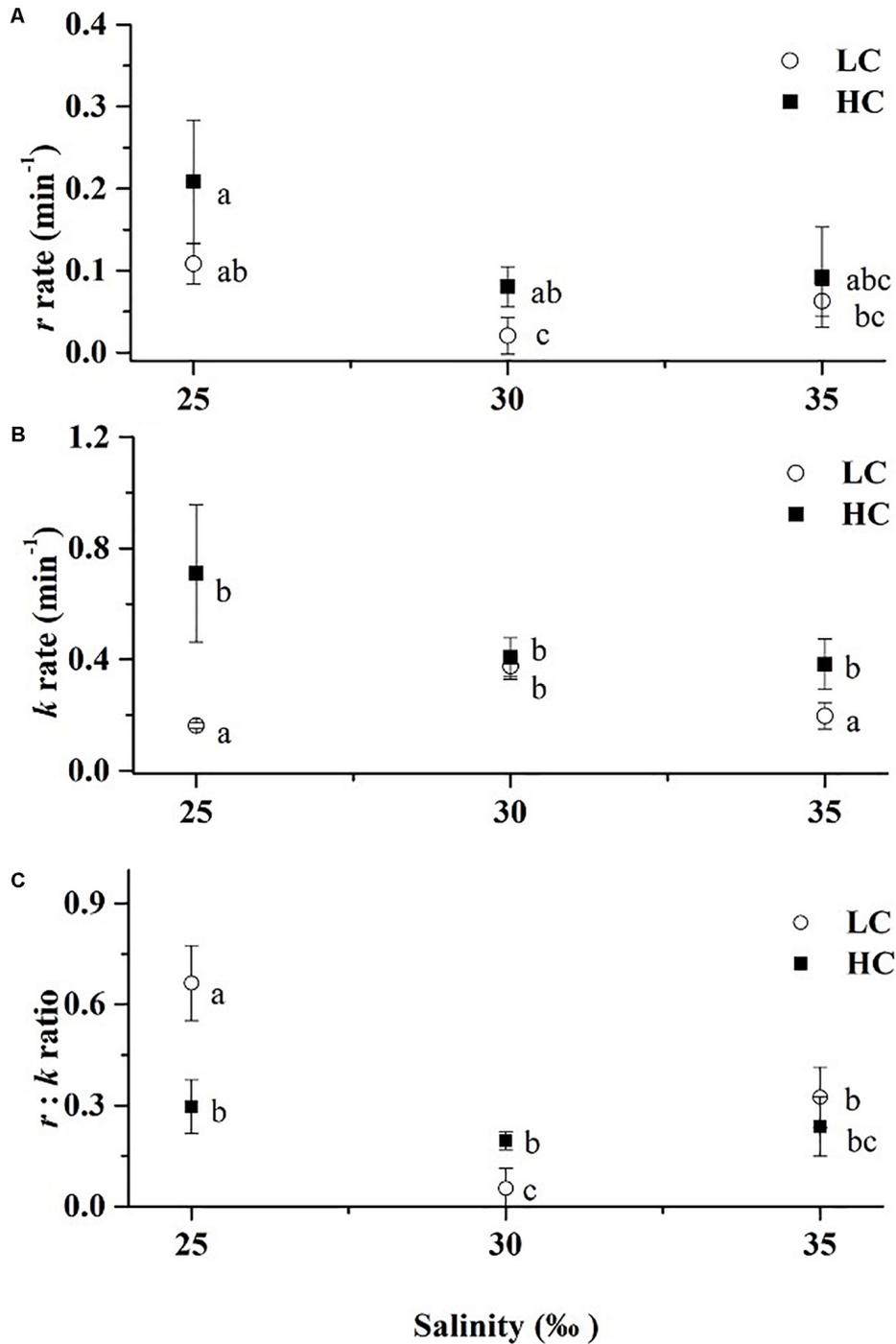


**FIGURE 5 |** Changes in effective photochemical quantum yield of cells grown under ambient (400  $\mu\text{atm}$ , LC, **A**) and elevated CO<sub>2</sub> (1000  $\mu\text{atm}$ , HC, **B**) when exposed to solar radiation for 48 min (800  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) under three salinity treatments: 25‰ (open triangles), 30‰ (half solid circles) and 35‰ (solid squares). Values are means  $\pm$  SD of triplicate cultures. Asterisks represent significant differences ( $p < 0.05$ ) among three salinity treatments.

increased almost linearly with decreasing salinity resulting in a marked increase in calcification:photosynthesis (C:P) at higher salinity, especially under HC. Lowered salinity (within the tested range) clearly interacted with  $p\text{CO}_2$  to synergistically enhance aspects of the physiological performance of *E. huxleyi* compared to each single driver alone.

Large populations of *E. huxleyi* are observed every summer in Norwegian coastal waters (Holligan et al., 1993; Wal et al., 1995), which is noteworthy considering that it is an essentially oceanic species with an offshore center of distribution (Winter et al., 1994). Paasche et al. (1996) inferred that *E. huxleyi*

may have developed distinct ecotypes in Scandinavian coastal waters. By coincidence, the strain in our study (PML B92/11) was originally obtained from coastal waters off Bergen, Norway with salinity of  $\sim 30\text{‰}$ , Mork et al., 1981). Here, although we cannot provide genetic data to infer the relationship to the strains discussed by Paasche et al. (1996), we have focused on the physiological performance of our strain under changing levels of salinity (25–35‰) and carbonate chemistry induced with elevated CO<sub>2</sub> (1000  $\mu\text{atm}$ ), and have shown that a reduction in salinity to 25‰ increased growth rate, effective photochemical efficiency and photosynthetic carbon fixation



**FIGURE 6 |** Rate of Repair *r* (A), damage *k* (B) and the ratio of repair:damage (C) after exposure to solar radiation for 48 min (800 μmol photons m<sup>-2</sup> s<sup>-1</sup>) under different treatments (LC, open circles; HC, solid squares). Values are means ± SD of triplicate cultures. Symbols with different lowercase letters indicate significant differences (*p* < 0.05) among different treatment.

even under ocean acidification (OA) conditions. Growth under HC caused a decrease in these parameters but, importantly, comparison between parameters under HC show that values at 25‰ were comparable to or even greater than those of cells

grown under 35‰, i.e., growth at lower salinity induced changes that ameliorated the negative effects of ocean acidification. For instance, photosynthetic rate under HC and 25‰ was 3.7 × the rate under 35‰ and HC although growth was

stimulated by only 9%. Even in comparison to values from cells grown under LC and 35‰, parameters in cells grown at 25‰ and HC showed similar values, except for growth rate, which was reduced by 9% and photosynthesis, which showed a 90% stimulation. While either OA or reduced salinity alone alters carbonate chemistry, salinity changes additionally influence ion concentrations, electrochemical gradients and osmolality, which modulate passive and active transport of CO<sub>2</sub> and nutrients and ultimately require extra energy. Photosynthetic carbon fixation rates of phytoplankton *in situ* at an estuary have been shown to increase with increased salinity below 25‰ but declined with salinity above that value, even though the dissolved inorganic carbon concentration increased (Li et al., 2011). In the present work, the result that *E. huxleyi* photosynthetically performs better under 25‰ than at higher salinity levels regardless of increased CO<sub>2</sub>, implies that this strain is more sensitive to salinity change than to changes of pCO<sub>2</sub>. Since increased levels of sunlight counteract the negative effects of OA on calcification in *E. huxleyi* (Jin et al., 2017), the tolerance to reduced salinity under OA could be attributed to its increased light capturing capability (Figures 2, 3) and enhanced photochemical performance.

*E. huxleyi* (PML B92/11) cells had a higher growth rate under reduced salinity when grown under ambient pCO<sub>2</sub> (LC, Figure 1A). This result is consistent with previous studies (Brand, 1984; Schouten et al., 2006), showing that strains isolated from coastal regions grew more rapidly under 25 than 33‰. Brand (1984) suggested that the last deglaciation was a possible selection time for coastal populations adapted for lower salinity. In the present study, since big differences in seawater carbonate chemistry exist between 25 and 35‰ (Table 1) as seen in the effects of salinity on CO<sub>2</sub> solubilities (Weiss, 1974) and DIC speciation, and the balance between photosynthesis (increasing with lower salinity) and calcification (unaffected by salinity), it might be argued that the increased CO<sub>2</sub> availability by up to about 39% were partially contributing to the enhanced growth (Figure 1) and changes in periplasmic redox activity related to osmotic pressure could be responsible for the enlarged cellular volume and stimulated growth under both CO<sub>2</sub> levels at lower salinity. However, the decrease in growth rates and photosynthetic rates at elevated CO<sub>2</sub> suggest that changes in pCO<sub>2</sub> are not responsible for the enhancement of these parameters under hyposaline conditions or are overridden by the greater H<sup>+</sup> concentration associated with OA, thereby decreasing the effect of salinity on growth under OA conditions.

*Emiliana* strains that have different calcifying capacities have different transport abilities for CO<sub>2</sub> and bicarbonate (Elzenga et al., 2000; Paasche, 2001; Stojkovic et al., 2013). The capacity of the strain used in this work to calcify has declined during long-term laboratory culture compared to that previously reported by another study (Sett et al., 2014), but is comparable with data reported by Jin et al. (2017) and Zhang et al. (2019) for the same strain. It has been suggested that low-calcifying strains are less efficient in utilizing bicarbonate for assimilation (Nimer and Merrett, 1992; Rost et al., 2003), though Stojkovic et al. (2013) showed the opposite with a greater proportion of DIC uptake from bicarbonate in low-calcifying strains and CO<sub>2</sub> use

only being more predominant in high-calcifying strains where bicarbonate is directed more to calcification.

While bicarbonate is the source of DIC used in calcification, and intracellular CO<sub>2</sub> is used for assimilation into organic carbon, the species of DIC taken up by coccolithophores is still contentious. *E. huxleyi*, in common with most other microalgae, possesses an active CO<sub>2</sub> concentrating mechanism (CCM) which is based on active transport of inorganic carbon across the plasmalemma and/or chloroplast envelope (Giordano et al., 2005). This usually involves the active uptake of bicarbonate (Taylor et al., 2011), but the acquisition of bicarbonate alone is subject to debate, given the evidence of Elzenga et al. (2000); Schulz et al. (2007), Bach et al. (2011); Stojkovic et al. (2013) and others that in addition to HCO<sub>3</sub><sup>-</sup>, CO<sub>2</sub> may be utilized (directly or indirectly via an external CA) by *E. huxleyi* as an external source of DIC for photosynthesis. It should, however, be borne in mind that the function of external CA is only to speed up the equilibrium between bicarbonate and CO<sub>2</sub> and does not in itself increase CO<sub>2</sub> concentrations above equilibrium values.

Although an enhanced growth rate of *E. huxleyi* (strains NZEH, AC472 and NIES 837) under elevated CO<sub>2</sub> concentrations has been reported before (Shi et al., 2009; Fiorini et al., 2011; Fukuda et al., 2014; Jin et al., 2017), in the present study the growth of *E. huxleyi* was lowered by 16–19% under the HC condition compared to the LC treatment, regardless of the salinity levels, which suggests that increased acidity could have stressed the cells (Figure 1A). Seawater acidification has also been shown to inhibit growth rate of *E. huxleyi* in other studies (Nimer et al., 1994; Hoppe et al., 2011). Ocean acidification has the dual effect of increasing pCO<sub>2</sub> but also increasing H<sup>+</sup> concentration, parameters that can have different effects on cellular performance (Hurd et al., 2020). Utilization of HCO<sub>3</sub><sup>-</sup> to provide CO<sub>2</sub> in photosynthesis generates OH<sup>-</sup>, driving the pH up, while calcification consumes OH<sup>-</sup>, driving the pH down. Since the PIC:POC ratio is seldom 1 (and is much lower than this in our experiments), acid-base regulation would require H<sup>+</sup> influx (Taylor et al., 2011; Raven and Crawford, 2012). The imbalance between photosynthesis and calcification as well as respiration may lead to the observed anomalous changes in the carbonate system between the beginning and end of the experiment at low salinity (Table 1).

The balance between a CO<sub>2</sub> rise and a decrease in pH is the key to reflect beneficial and harmful effects of OA, but such a balance can be perturbed by other environmental drivers (see the review by Gao et al., 2019 and literature therein). In the present work, lowered salinity from 35 to 25‰, which would result in slightly higher CO<sub>2</sub> solubility but markedly lower HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup> concentrations, antagonistically acted with OA to influence the growth of *E. huxleyi*.

The photosynthetic rates, in accordance with the growth rate, increased with decreased salinity under both LC and HC (Figure 4A). Such a relationship in photosynthesis was more obvious than in the specific growth rate (Figures 1, 4). Since the mitochondrial respiration rate of *E. huxleyi* is enhanced under OA treatment (Jin et al., 2015), the balance between photosynthesis and respiration can reasonably be reflected in the differential responses between growth and photosynthesis.

Therefore, reduced salinity with the increased availability of CO<sub>2</sub> would have resulted in higher photosynthetic rates, though increased acidity from higher CO<sub>2</sub> exacerbated negative impacts as reflected in differences between the growth rates of the HC and LC-grown cells (**Figure 1**). While differences of [CO<sub>2</sub>] between 25 and 35‰ media were much smaller (**Table 1**) compared to that between LC and HC, osmotic regulation with enhanced energy supply due to increased light capture and use efficiency (**Figures 2, 3**) could facilitate assimilation of biogenic elements. On the other hand, changes in salinity might indirectly alter the efficiency of CCMs (Booth and Beardall, 1991; Goyal et al., 1992; Giordano et al., 2005) due to its effects on inorganic carbon levels in the bulk medium around algae, and altered CCM efficiency may make the discrepancies in intracellular [CO<sub>2</sub>] far larger than those of the surrounding medium (Rost et al., 2003; Schulz et al., 2007), although activity of the CCMs of *E. huxleyi* are supposed to be low (Rost and Riebesell, 2004; but see Stojkovic et al., 2013). Though CCMs are not completely down-regulated under high pCO<sub>2</sub> conditions, induced by OA or lower salinity, the passive diffusion of CO<sub>2</sub> may make a bigger contribution to photosynthesis as the increased CO<sub>2</sub> under the experimental OA environment partially compensates for the reduced internal CO<sub>2</sub> due to reduced CCM activity. The physiological benefit of such a downregulation of CCM activity is the energetic savings from running the energy-expensive CCM (Raven et al., 2014) at a lower level. Furthermore, a higher photosynthetic rate under reduced salinity (25 and 30‰) might have enhanced osmoregulation, as many organic osmolytes are typically photosynthetic products (Kirst, 1990).

In our study, cells grown under HC conditions had lower photosynthesis rates irrespective of salinity treatments (**Figure 4A**). The negative effects of low pH ( $\leq 7.7$ ) could have offset the positive effects of increasing [CO<sub>2</sub>] (about twice that of LC medium, **Table 1**) as previous studies show that higher intracellular [H<sup>+</sup>] would directly result in a decreased pH in the stroma of the chloroplast, which then led to reduced CO<sub>2</sub> fixation (Werdan et al., 1975; Coleman and Colman, 1981; Suffrian et al., 2011). In addition, HC treatment might, at least partially, down-regulate the expression of CCMs, energetically costly mechanisms to increase intracellular CO<sub>2</sub> at the active site of Rubisco (Raven et al., 2014). Evidence from diatoms (Burkhardt et al., 2001) and the marine cyanobacterium *Trichodesmium* (Hutchins et al., 2007) suggests that CCMs in these species are close to full downregulation at 1000–1500 p.p.m. CO<sub>2</sub>. The decrease in activity of CCMs thus may result in lower intracellular [CO<sub>2</sub>] and reduced photosynthetic rate compared to LC, as reflected in a diatom and macroalgae (Xu and Gao, 2012a; Liu et al., 2017), though energy saving and passive diffusion from a higher bulk seawater pCO<sub>2</sub> could partially compensate for the reduced intracellular [CO<sub>2</sub>].

Calcification rates of *E. huxleyi* are known to be sensitive to changes in seawater carbonate chemistry. As HCO<sub>3</sub><sup>-</sup> is the main substrate for calcification (Buitenhuis et al., 1999; Paasche, 2001; Roleda et al., 2012), the increasing calcification rates of cells grown under HC with increasing salinity in our study (**Figure 4B**) could be the result of increased [HCO<sub>3</sub><sup>-</sup>] concentration (**Table 1**). However, there were no significant

differences in calcification rates among three salinity treatments under LC with even a slightly higher value at 25‰ (**Figure 4B**), which is consistent with Beaufort et al. (2011), who also showed a weak correlation between calcification and salinity in their field work. Under LC, the highest effective photochemical quantum yield values and *r:k* ratios among the three salinity treatments were observed under 25‰ after an exposure to high light (**Figures 5A, 6C**), suggesting a relatively thick coccolith layer playing a protective role (Xu and Gao, 2012b), which is in accordance with the measured results (**Figure 4B**). Nimer and Merrett (1993) proposed that the optimum pH for calcification was 7.8, a value just close to the pH of our medium under 25‰ and LC conditions (**Table 1**), so that less energy would be required to maintain intracellular acid-base balance. Sikes and Wilbur (1982) demonstrated that, when salinity was reduced from 34.5 to 19.5‰, the percentage of calcified cells increased in the medium. Taylor et al. (2011) identified a plasma membrane H<sup>+</sup> channel that played an important role in pH homeostasis in coccolithophores. Both rectifying H<sup>+</sup> channels and rectifying Cl<sup>-</sup> channels worked together to sustain H<sup>+</sup> efflux. In our study, the lower concentration of Cl<sup>-</sup> associated with the reduced salinity in the 25‰ medium implies that Cl<sup>-</sup> efflux was easier to carry out and assist H<sup>+</sup> removal to keep the intracellular pH stable, which could ultimately have promoted calcification.

There has been controversy in the past about how increased pCO<sub>2</sub> in seawater affects calcification of *E. huxleyi*. Calcification rates of *E. huxleyi* (PML B92/11) at high pCO<sub>2</sub> appeared to be dependent on pH (Bach et al., 2011). In the present work, elevated pCO<sub>2</sub> along with lowered pH decreased the calcification of *E. huxleyi*, which is in accordance with previous work (see the review by Gao et al., 2019 and references therein). However, in other studies, increased pCO<sub>2</sub> along with decreased pH increased calcification of *E. huxleyi* (Iglesias-Rodriguez et al., 2008) or had no significant effect on it (Langer et al., 2009). It seems that responses of calcification of *E. huxleyi* are likely to be strain-specific (Langer et al., 2009). The decreased calcification could be a strategy to reduce cytosol acidification (Feng et al., 2017) as well as a maneuver to save energy expended in acid/base regulation. However, such HC-induced impacts became almost absent in the cells grown under a salinity of 35‰ (**Figure 4B**). Under LC, it may be easier for the cells to remove H<sup>+</sup> (Mackinder et al., 2010), while under HC the concentration of HCO<sub>3</sub><sup>-</sup>, the substrate for calcification, was higher (**Table 1**). Previous studies have suggested that bicarbonate concentrations may not saturate the requirement of *E. huxleyi* for calcification as the K<sub>1/2</sub> (HCO<sub>3</sub><sup>-</sup>) values have been reported at 3256 μM (Buitenhuis et al., 1999), a value far removed from the current and predicted future high pCO<sub>2</sub> ocean. Under this consideration, any induced promotion in bicarbonate concentrations could favor calcification. With increasing salinity, the proportion of HCO<sub>3</sub><sup>-</sup> increases (Weiss, 1974; **Table 1**), and thus led to a slightly higher calcification rate under 35‰ and HC conditions (**Figure 4B**), which is consistent with the result reported by Fukuda et al. (2014). This finding helps to enhance our understanding as to why coccolithophores were able to thrive in the early Mesozoic era, a time that was characterized by relatively low sea water pH (as low as pH 7.7) and high DIC concentrations (Ridgwell, 2005).

From the ratio of calcification to photosynthesis (C:P), we could see different strategies cells adopted when they grew under different carbonate chemistry conditions. A quite low value for this parameter ( $<0.2$ ) under 25 and 30‰, regardless of  $p\text{CO}_2$  (Figure 4C), indicates that organic carbon production was prioritized over inorganic carbon production at both DIC levels. In contrast, the C:P ratio was far higher ( $>0.3$ ) under 35‰ (Figure 4C), suggesting that cells could “invest” more inorganic carbon to secrete coccoliths if the DIC supply was increased (Table 1). The results of an additional experiment (for details see Supplementary Materials) clearly provided evidence for such behavior: when the salinity was set at 25‰ with elevated DIC concentration, its C:P ratio significantly increased (Supplementary Figure S1). This result agreed with previous findings with qRT-PCR analysis (Bach et al., 2013), showing that some genes with putative roles in DIC,  $\text{Ca}^{2+}$  and  $\text{H}^+$  transport (*AEL1*, *CAX3*, and *ATPVC/c*) may play a dual role, supporting calcification under ambient conditions but switching to support photosynthesis when DIC becomes limiting.

Our results suggest that *E. huxleyi* PML B92/11 has the ability to acclimate to different levels of salinity, pH and  $p\text{CO}_2$ , by up-regulating its photosynthetic performance with insignificant changes in calcification. *E. huxleyi* is known to grow well even with reduced calcification, or in non-calcifying strains, under OA conditions (Jin et al., 2017). Our findings, along with other reported results, explain its success in coastal waters, an environment characterized by acute changes in, or fluctuation of, pH/ $p\text{CO}_2$  and salinity. Tyrrell et al. (2008) reported that *E. huxleyi* could only survive in natural waters with salinity above 11. We found that when salinity was decreased to 15‰, cells of this strain could barely survive, with non-measurable growth (data not shown). Since sensitivity of different strains of *E. huxleyi* to ocean acidification differs substantially (Langer et al., 2009), the combined effects of OA and salinity could therefore be different in different regions or under different hydrological processes.

Under the future scenario of ocean acidification with decreased salinity, the tolerance of *E. huxleyi* shown in this study will increase its competitiveness and help it better adapt

to future global environmental changes, especially in the coastal areas of high latitude. A potential niche extension of *E. huxleyi* into higher latitude waters driven by polar ice melt and progressive OA might then influence the distribution of other phytoplankton and have profound consequences for the ocean's carbon cycle. Thus, further studies that could better mimic the future scenario are needed.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

## AUTHOR CONTRIBUTIONS

JX and KG designed the experiments. JX carried out the experiments. All authors contributed to data analysis and manuscript writing.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2020.00704/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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