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Significance of HCO_3^- alkalinity in calcification and utilization of dissolved inorganic carbon in *Chara vulgaris*

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ABSTRACT: To investigate the influence of HCO_3^- alkalinity on calcification in thalli of the genus *Chara*, we studied the effects of increased light level and additional HCO_3^- on calcification rate in *C. vulgaris* at various hours (30 min, 8 h, 12 h, 12.5 h, 20 h and 24 h after treatment) in a 24 h experiment (12 h light:12 h dark). We identified a significant Pearson's correlation between exogenous dissolved inorganic carbon (DIC) concentrations and the utilization of DIC (µmol C g^{-1} fresh weight). Plotting the daily rhythm of DIC utilization produced negative quadratic curves. Furthermore, calcification rate (µmol Ca h^{-1} g^{-1}) was linearly related to DIC utilization rate (DIC_{uptake}; µmol C h^{-1} g^{-1}), indicating that the calcification rate is dependent on DIC_{uptake}. However, ratios of calcification to the utilization of DIC were decreased at high light intensity and increased with HCO_3^- addition, which was mainly ascribed to changes in the ratio of calcification to photosynthesis. Chlorophyll fluorescence results provided direct evidence for the promotion of photosynthesis in *Chara* thalli by both high light and DIC addition and their positive influence on maximum relative electron transport rate. These results suggest that calcification in calcareous *C. vulgaris* is mainly restrained by HCO_3^- alkalinity, which could explain the correlation between calcification of *Chara* thalli and alkalinity of water bodies in the field.

KEY WORDS: Calcification · Dissolved inorganic carbon utilization · Rapid light curves · *Chara* · Calcification · Photosynthesis

INTRODUCTION

$CaCO_3$ encrustations are common on the stems, branchlets, and surfaces of oogonia in *Chara* spp. (hereafter '*Chara*') (Wood & Imahori 1965). However, intra- and inter-specific variations in the external encrustations on the cell wall are present within this genus (Anadón et al. 2002), as well as between ecorticate species and corticated species (Kawahata et al. 2013). Furthermore the average percentage content of encrustation in dry charophyte mass varied from 15% (Siong & Asaeda 2009a) to 60% (Hutchinson 1975), 61.5% (Kufel et al. 2013), 70% (Blindow 1992), and 77% (Urbaniak 2010), depending on the studied species and the water environment where those species were collected. However, carbonate deposited by dense charophyte vegetation accounts for a significant portion of calcium carbonate in the sediments of so-called *Chara* lakes and represents an important record of environmental conditions (Apolinarska et al. 2011, Pelechaty et al. 2013, Kufel & Strzałek 2016).

Light and dissolved inorganic carbon (DIC) are 2 of the most important environmental variables that affect the morphology and distribution of *Chara* plants

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(McConnaughey 1998) because of their fast attenuation and limited solubility in the water column, respectively. In natural habitats, most calcareous species of *Chara* inhabit waters of relatively high alkalinity and calcium content (Kufel & Kufel 2002). Obvious encrustation has been observed under high light intensity (Wang 2008). However, there is much debate on the influence of light and HCO_3^- alkalinity on calcification. According to the cis-calcification model, calcification on alkaline surfaces of *Chara* is a by-product of bicarbonate assimilation from alkaline water, and light therefore promotes calcification (McConnaughey & Whelan 1997). However, in the trans-calcification model, Ca^{2+}–$2H^+$ exchange catalysed by Ca^{2+} ATPase is responsible for calcification in *Chara* (McConnaughey & Falk 1991). Therefore, in the trans-calcification model, calcification does not require light, and calcification functions as a proton generator. Since the 1990s, many studies have demonstrated the beneficial effects for macrophytes from proton generation by calcification and how this is related to bicarbonate utilization (McConnaughey 1991, McConnaughey & Falk 1991), nutrient assimilation (McConnaughey & Whelan 1997, Siong & Asaeda 2006, 2009b, Gomes & Asaeda 2010, Wang et al. 2013), and hyper-accumulation of heavy metals (McConnaughey 1991, Siong & Asaeda 2009a). Furthermore, Wang et al. (2013) demonstrated that calcification could mitigate the toxic effects of high NH_4-N on calcareous *Chara* plants.

In mildly alkaline water, DIC is typically decreased in 2 ways, either by uptake through the photosynthesis of algae or by becoming incorporated into the cell walls of calcareous *Chara* by calcification (Ray et al. 2003). Owing to the sound understanding of the relationships between fluorescence parameters and photosynthetic electron transport *in vivo* (Maxwell & Johnson 2000, Baker 2008), fluorescence parameters such as photosynthetic efficiency estimates (F_v/F_m) have been widely used to assess the photosynthetic characteristics of submerged plants or macroalgae (Schwarz et al. 2000 and Durako & Kunzelman 2002 for seagrass, Beer & Axelsson 2004 for macroalgae, Asaeda et al. 2014 for *Chara* spp.). In addition, the rapid light curve (RLC) is a powerful tool for the assessment of photosynthetic performance and for providing detailed information on the saturation characteristics of electron transport (Ralph & Gademann 2005).

C. vulgaris L. is a polymorphic charophyte with a worldwide distribution between 70° N and 50° S. *C. vulgaris* is prone to accumulate lime and form apparently non-banded encrustation on its stems (Anadón et al. 2002), accounting for more than 60% of ash mass under 50% of sunlight (Wang et al. 2008). To clarify the influence of light and HCO_3^--alkalinity on calcification, we investigated the effects of high light intensity and HCO_3^- addition on the calcification rate of *C. vulgaris* by using an open experimental system. Furthermore, RLC was used to assess the effects of light level increase and HCO_3^- addition on photosynthetic performance. The aims of this study were to: (1) investigate the occurrence of dark calcification by monitoring the daily rhythm of the calcification rate of *Chara* thalli under constant light and (2) identify the rate-limiting factor for calcification of calcareous plants.

MATERIALS AND METHODS

Plant culture

On 5 April 2010, 24 clusters of shoots (10–15 cm in length) of *Chara vulgaris* were collected from a depth of 1–1.5 m in Liangzi Lake (30° 05′ to 30° 18′ N, 114° 21′ E to 114° 39′ E), a shallow lake located in the middle reach of the Yangtze River in China. These specimens were then transplanted into 3 cylindrical aquaria (1 m in radius) located at Donghu Experimental Station of Lake Ecosystems, Institute of Hydrobiology, Chinese Academy of Sciences. These aquaria were filled with tap water and sediment from the Tanglinhu area, as described by Feng et al. (2006). After 1 mo, the shoots of the plants had developed towards the water surface, with a branched morphology and visible calcified encrustation on the internodes. On 15 May 2010, more than 120 apical tips with 3 or 4 internodes (about 4–5 cm in length) were cut using scissors and pre-incubated in 1 l glass beakers (n = 4) filled with plant-containing water (PCW). To avoid the effect of Ca^{2+} and Mg^{2+} contents on calcification, PCW was obtained by filtering water from the previously used circle aquaria through GF/C membranes (Whatman), with DIC content of 0.98 ± 0.04 mM C, Ca^{2+} content above 60 mg l^{-1} and Mg^{2+} content below 30 mg l^{-1} (McConnaughey 1998, Kufel & Kufel 2002, Gomes & Asaeda 2010, Asaeda et al. 2014). All beakers were placed in the plant growth incubator under controlled conditions: temperature, 25 ± 2°C; illumination on the water surface, 90 µmol m^2 s^{-1}; and a photoperiod of 12:12 h (light:dark). After 2 wk of pre-culture, inorganic carbon accumulated within the plants was removed by transferring all apical tips to 1 l beakers containing ultrapure water for 6 h under the same culture conditions (Kahara & Vermaat 2003).

Experimental design

A factorial design of 2 × 2 of light intensity and DIC level was used in this experiment: low light (LL, 90 µmol m^{-2} s^{-1}) and high light (HL, 150 µmol m^{-2} s^{-1}) intensities and low DIC (LC: no HCO_3^- addition) and high DIC (HC: 1 mM HCO_3^- addition) levels. These 4 combined treatments were marked as LL-LC, LL-HC, HL-LC, and HL-HC, respectively. For each treatment, 24 flasks (4 flasks each time × 6 times) with plants were used as replicates, and 12 flasks (2 flasks each time × 6 times) without plants were used to calibrate changes to the solution over time. At the beginning of the photoperiod (Time 0), 4 or 5 apical tips (about 0.5 g in fresh weight, without endogenous inorganic carbon) were weighed and transferred into 96 flasks (24 flasks × 4 treatments) containing 200 ml of the culture solution. In all, 144 conical flasks (96 flasks + [12 flasks × 4]) with a capacity of 250 ml were placed in 2 plant incubators with different light levels (each contained 72 flasks), with the same temperature conditions as for pre-culture. The addition of HCO_3^- was made by adding $NaHCO_3$, and the pH of the culture solutions was adjusted to 8.2 by using 0.1 M NaOH (before being decanted into the flasks).

Experimental sampling and measurement

To study the utilization of DIC and calcification rate during the day and night, at various hours (30 min, 8 h, 12 h, 12.5 h, 20 h and 24 h after treatment), 20 ml of the solution were removed and stored at −20°C (10 ml for DIC analysis and 10 ml for ion content measurement). Four flasks with plants and 2 flasks without plants were used for each point of the daily cycle. The DIC content of the solution was measured using a total organic carbon analyser (O.I. analytical 1010). Analyses of cation content were performed using a Dionex DX100 ion chromatograph equipped with a 25 µl sample loop, a cation-exchange column and a suppressed conductivity detection system. For separation, an ion Pac CG10A guard column (50 × 4 mm) was coupled to an IonPac CS10A analytical column (250 × 4 mm). A Dionex CSRS ultra self-regeneration suppressor was employed, and the 2.0 software was used for system control and data acquisition. All IC-related equipment was supplied by Dionex.

Pulse-amplitude modulated (PAM) chlorophyll fluorescence parameters were monitored using a submersible PAM fluorometer (diving-PAM; Walz). RLCs were generated for branchlets mounted in the chamber. According to the procedure of Ralph & Gademann (2005), RLCs were generated using a pre-installed software routine, where the actinic light was increased in 8 steps (plus initial quasi-darkness measurement) beginning at an initial irradiance for a prescribed duration. To allow relative electron transport rate (rETR) to reach a plateau (over at least 2 points) and decline during the last light steps, 8 consecutively increasing actinic light intensities of 3, 16, 42, 77, 125, 187, 339 and 501 µmol m^{-2} s^{-1} were used. The first saturating pulse occurred after the quasi-darkness period. The second to ninth measurements occurred after a stepped actinic irradiation of 10 s duration, from 3 to 501 µmol photons m^{-2} s^{-1}. An RLC takes approximately 90 s and records fluorescence yield (F), maximum fluorescence yield of a light-adapted leaf (F'_m), effective quantum yield of PSII (ΦPSII), and ETR, which can be plotted as a function of photosynthetically active radiation (PAR) irradiance. To quantitatively compare RLCs under different treatments, they were described by several character parameters: α (photosynthetic rate in the light-limited region of RLC), Ek (minimum saturating irradiance) and rETRm (maximum rETR), determined by non-linear regression with the empirical equation proposed by Platt et al. (1980).

Calculation and analysis

DIC uptake by plants in each sample was calculated as the difference of DIC content in the solution with plants and the means of DIC content in 2 flasks without plants. A similar approach was used for Ca^{2+} uptake. To avoid the effect of the pre-experiment fresh weight of plants, utilization of DIC at each sampling time was expressed as µmol C g^{-1} fresh weight, while calcification was expressed as µmol Ca g^{-1} fresh weight. To study the daily rhythm of calcification, calcification rate was defined as µmol Ca h^{-1} g^{-1} of fresh weight (Ca^{2+}_{uptake}); similarly, utilization rate of DIC was expressed as µmol C h^{-1} g^{-1} of fresh weight (g) (DIC_{uptake}).

Regression curves were used to express the time course of DIC concentration in the solution. The effects of HCO_3^- addition and light increase on the Ca^{2+} and DIC contents of the solution were tested using 2-way ANOVA. Pearson's correlation was used to analyse the relationship between utilization of DIC and DIC concentration, as well as calcification and DIC concentration in solution. Inverse regression

curves were used to express the time course of Ca^{2+}_{uptake}. The correlation between DIC_{uptake} and Ca^{2+}_{uptake} was calculated by linear regression. Nonlinear regression was used to determine the character parameters of RLC, and the effects of DIC addition and light increase on the above parameters were evaluated by 2-way ANOVA. All data were analysed using SPSS 13.0.

RESULTS

Effect of plants on DIC concentration in the solution

As expected, plant photosynthesis and calcification significantly affected the DIC concentration of the solution. The variation of DIC concentration in the solution across sampling times for all treatments could be fitted to a quadratic equation, i.e. DIC concentration sharply decreased during the light period but slowly increased during the dark period (Fig. 1). The results of 2-way ANOVA revealed that both light levels and the addition of HCO_3^- significantly affected the contents of DIC at 12 and 24 h sampling times (Table 1). The largest change in DIC was found in LL-HC at 12 h and in HL-HC at 24 h (Fig. 1). Addition of HCO_3^- significantly decreased the pH of the solution, therefore ΔpH at low HCO_3^- levels was higher than that at high HCO_3^- levels (Table 1).

Variation in DIC utilization and calcification of *Chara* thalli

DIC utilization (μmol C g^{-1}) followed a negative quadratic equation with sampling time (Fig. 2). Pearson's correlation showed that DIC utilization (μmol C g^{-1}) was significantly negatively related to DIC concentration for each treatment, whereas significant correlation between calcification (μmol Ca g^{-1}) and DIC concentration only occurred under low light conditions (Table 2).

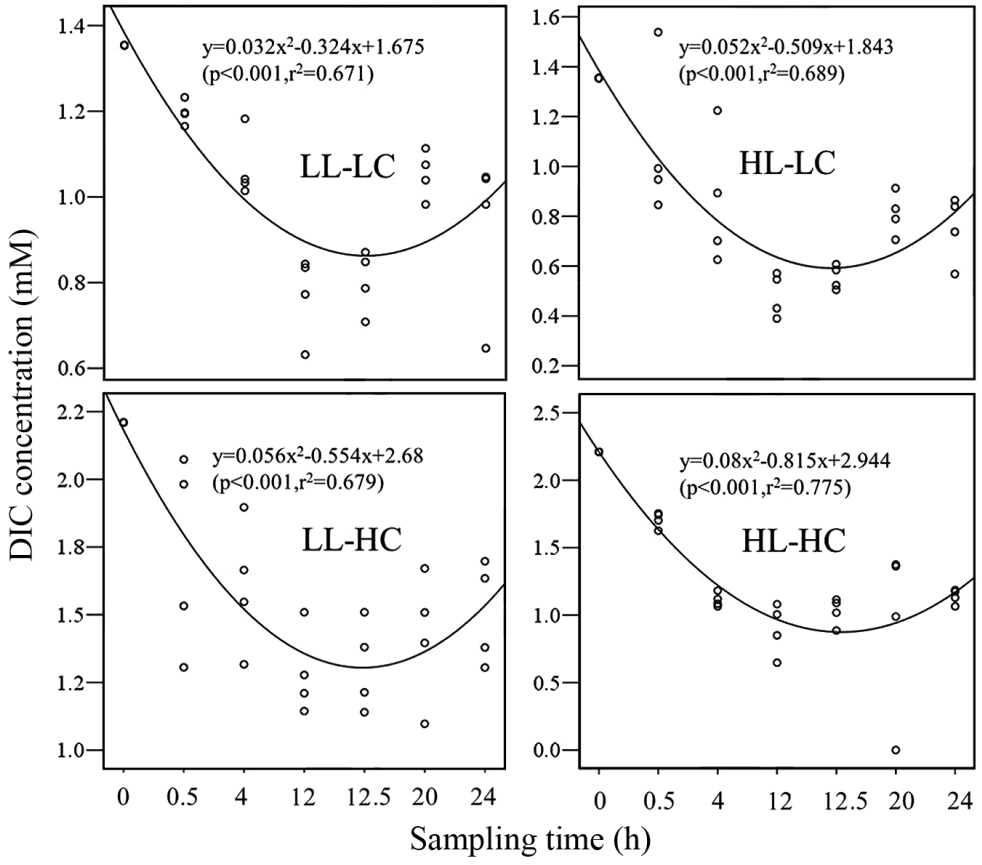

Fig. 1. Variations in the concentration of dissolved inorganic carbon (DIC) in solution with sampling time in 4 different treatments: LL-LC, LL-HC, HL-LC and HL-HC, where LL: low light (90 μmol m^{-2} s^{-1}), HL: high light (150 μmol m^{-2} s^{-1}), LC: low DIC (no HCO_3^- addition), HC: high DIC (1 mM HCO_3^- addition)

Table 1. *F*-values and significance of 2-way ANOVA of the effects of dissolved inorganic carbon (DIC) and light level on the ratio of calcification to utilization of DIC, increased pH and variation in DIC both at 12 and 24 h for *Chara vulgaris* thalli. ***p < 0.001, **p < 0.01, *p < 0.5, ns: p > 0.5

Parameter	DIC (D)	Light (L)	D × L
Ratio of calcification to utilization of DIC (slopes of lines)	8.051*	18.630***	5.804*
ΔpH	21.544**	2.606 ns	0.017 ns
Variation in DIC at 12 h	9.827**	27.777***	4.612 ns
Variation in DIC at 24 h	42.529***	1.758 ns	20.419***

The highest calcification rate (Ca^{2+}_{uptake}) was found at 0.5 h, with sharp decreases in Ca^{2+}_{uptake} at all subsequent time points for all treatments. An inverse regression demonstrated a significant decrease in Ca^{2+}_{uptake} over time (Fig. 3). HL decreased Ca^{2+}_{uptake}, whereas the addition of HCO_3^- increased Ca^{2+}_{uptake}. The calcification rate was linearly related to the utilization rate of DIC for all treatments (Fig. 4). Ratio of calcification rate to utilization of DIC rate, i.e. slopes of lines shown in Fig. 4, was higher at HC than at LC, while it was lower at HL than that at LL levels (Table 1, Fig. 4).

Responses of chlorophyll fluorescence to light levels and addition of HCO_3^-

All plots of ETR as a function of irradiance showed the classical shape of a photosynthesis–irradiance (P–E) curve, with a linear rise in the light-limited region followed by a plateau, where PAR is above Ek and the photosynthetic pathway becomes limited (Fig. 5a). Both HL and HC significantly increased rETRm and Ek (minimum saturating irradiance). There was a significant interaction on rETRm between light level and DIC addition (Table 3). The highest rETRm was found in HL-HC (Fig. 5b).

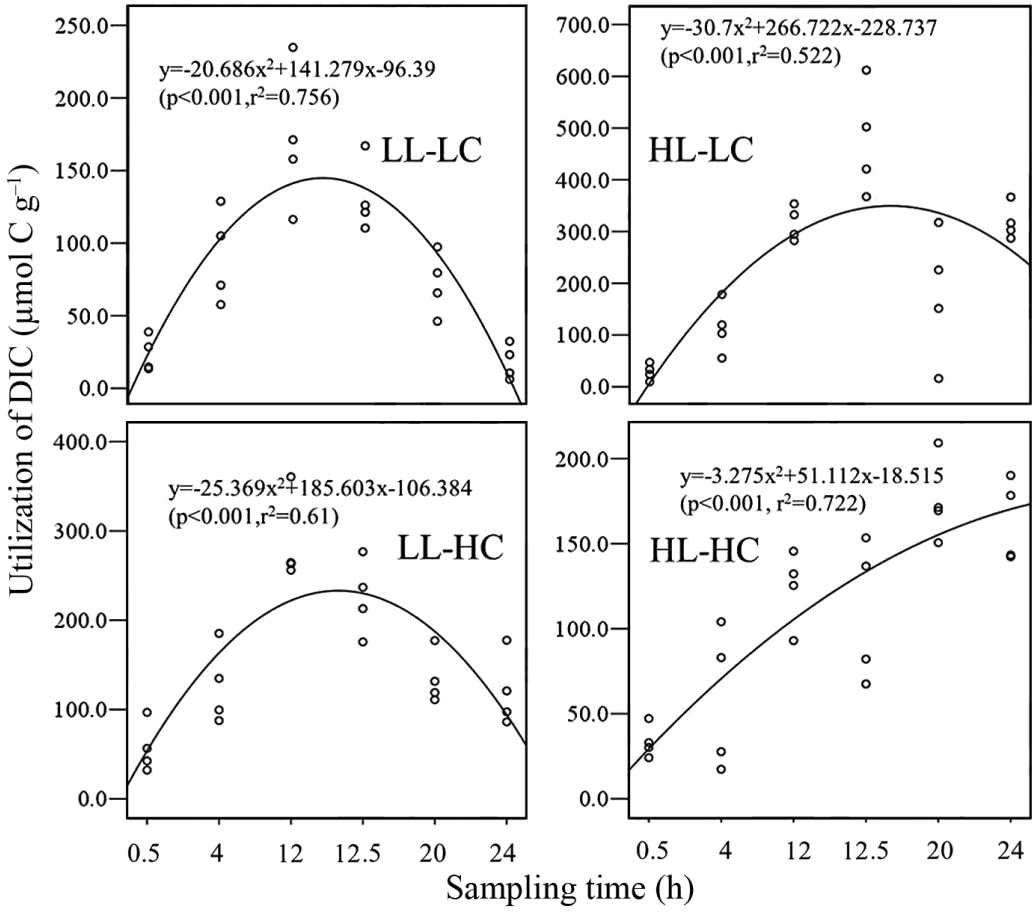

Fig. 2. Variations in the utilization of dissolved inorganic carbon (DIC; μmol C g^{-1}) by thalli of *Chara vulgaris* with sampling time in 4 different treatments: LL-LC, LL-HC, HL-LC and HL-HC. Treatments are defined in Fig. 1

Table 2. Coefficient and significance of Pearson's correlation between utilization of DIC (µmol C g^{-1}) and DIC concentration (µmol C l^{-1}), as well as between calcification (µmol Ca g^{-1}) and DIC concentration in solution under 4 different treatment conditions. *p < 0.05(2-tailed); **p < 0.01

	LL-LC	LL-HC	HL-LC	HL-HC
Utilization of DIC	−0.498*	−0.536**	−0.780**	−0.427*
Calcification	−0.642*	−0.738**	−0.339	−0.320

DISCUSSION

Daily rhythm of the calcification rate

To date, no experiment under constant light intensity has addressed the daily rhythm of calcification of *Chara* thalli. Although many studies have been performed on coral calcification, revealing non-constant daily patterns of calcification rate, the results exhibited many specific-species differences (e.g. Moya et al. 2006, Al-Horani et al. 2007). Here, we found that under the constant light intensities of 90 and 150 µmol photons m^{-2} s^{-1}, the calcification rate of *Chara* thalli showed a continuous decrease during a 24 h period (light:dark, 12:12 h) at both DIC levels tested (Fig. 3). According to McConnaughey (1998), the calcification rate of *Chara* thalli is mainly affected by light intensity and Ca^{2+} and DIC concentrations in the solution. In the present study, to avoid the effect of Ca^{2+} and Mg^{2+} contents on calcification, Ca^{2+} content in the solution was above 60 mg l^{-1}, while Mg^{2+} was below 30 mg l^{-1} during the experiment (McConnaughey 1998, Kufel & Kufel 2002, Gomes & Asaeda 2010, Asaeda et al. 2014), and the light intensity did not change during the light period. Therefore, the effects of light intensity and Ca^{2+} and Mg^{2+} contents in the solution on photosynthesis and calcification were negligible. Thus, the observed decrease in calcifi-

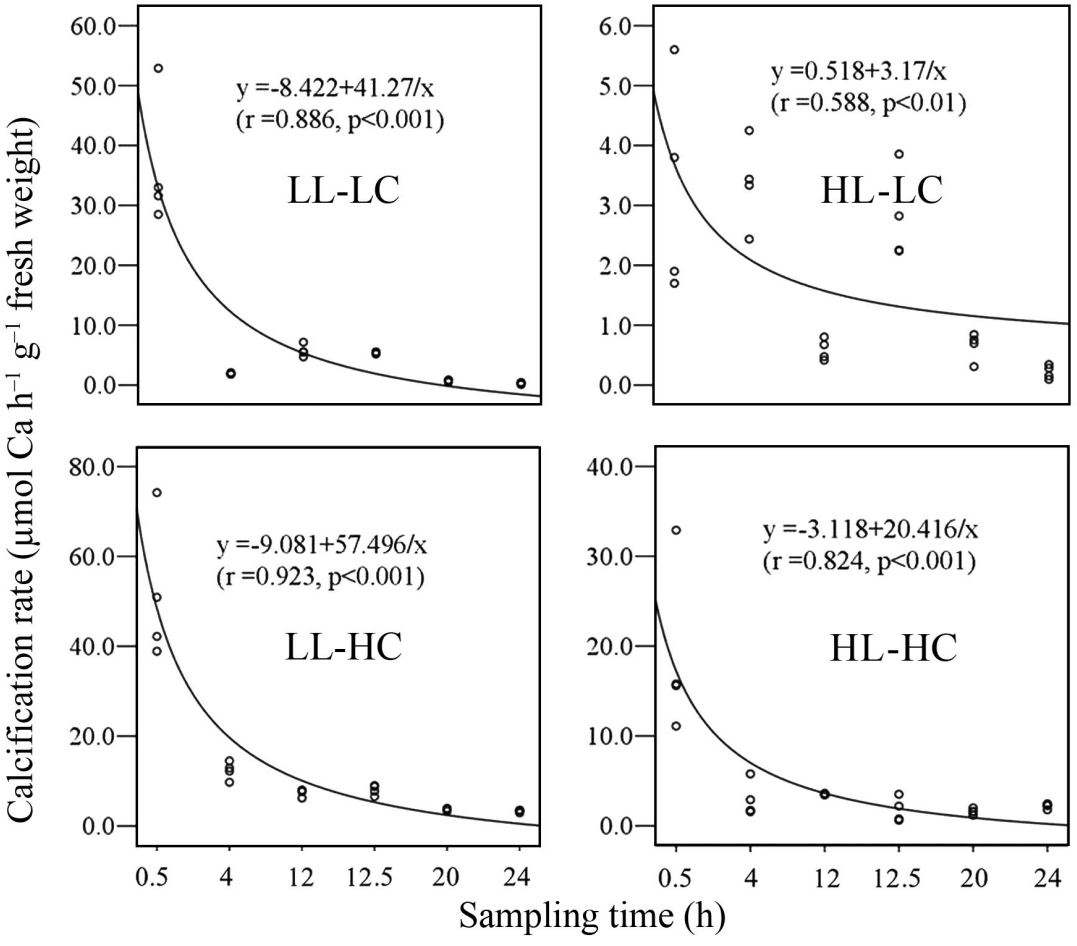

Fig. 3. Variations in calcification rate (Ca^{2+}_{uptake}; µmol Ca h^{-1} g^{-1}) by thalli of *Chara vulgaris* with time in 4 different treatments: LL-LC, LL-HC, HL-LC and HL-HC. Treatments are defined in Fig. 1

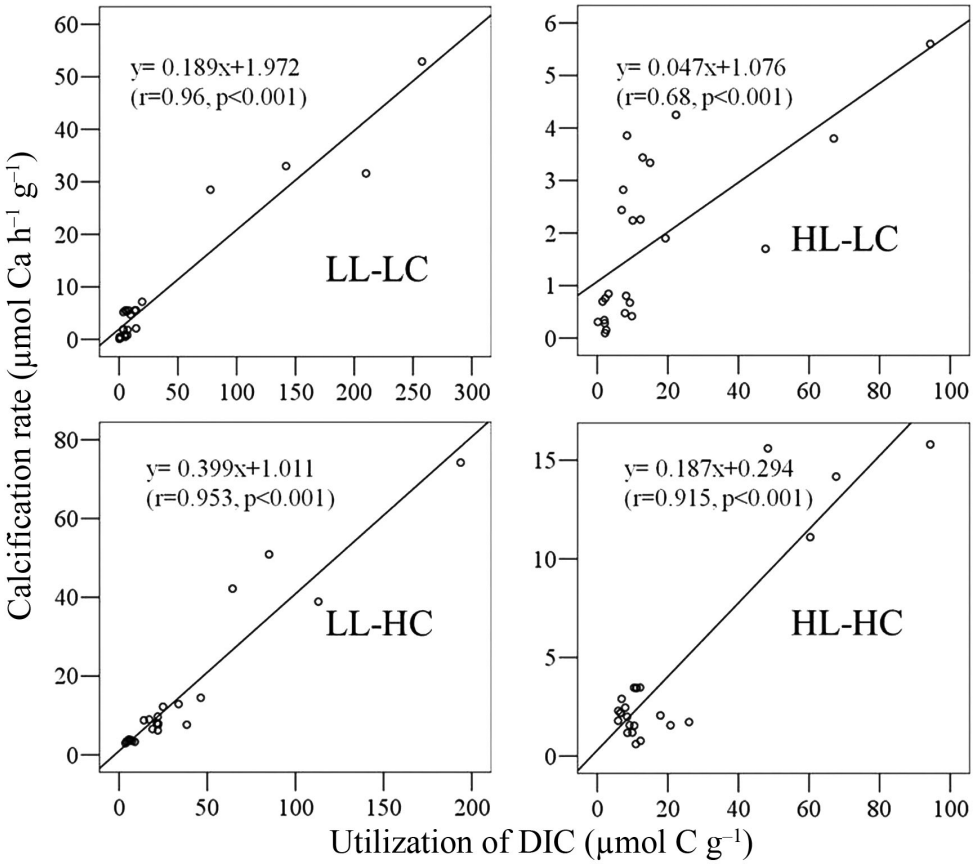

Fig. 4. Correlations between calcification rate (Ca^{2+}_{uptake}) and the utilization rate of dissolved inorganic carbon (DIC_{uptake}; μmol C h^{-1} g^{-1}) for thalli of *Chara vulgaris* in 4 different treatments: LL-LC, LL-HC, HL-LC and HL-HC. Treatments are defined in Fig. 1

cation rate should be ascribed to a restriction in DIC concentration. This is further supported by a significant negative Pearson's correlation between calcification and DIC concentration under LL conditions and the much lower calcification rate found in the HL-LC treatment (Fig. 3). Our finding that external DIC concentration significantly affects the calcification rate of *Chara* thalli differs from the findings of previous studies that reported endogenous (plant body) inorganic carbon being used in the calcification of *Chara* (McConnaughey 1991). In addition, many studies of coral have shown that calcification is independent of exogenous DIC concentration (e.g. Furla et al. 2000). However, this independence of calcification on DIC content in the solution can be explained by the presence of sufficient inorganic carbon within the plants. In this study, endogenous inorganic carbon was exhausted during pre-treatment. Thus, external HCO_3^- showed a significant effect on the calcification rate of the plants.

Dark calcification

According to the trans-calcification models proposed by McConnaughey & Whelan (1997), *Chara* calcification does not require light. However, to date no experimental study has provided direct data on dark calcification of *Chara*. In the dark, plant photosynthesis ceases completely and CO_2 is produced by respiration; therefore, DIC uptake of the plants (calculated as the difference of DIC content in the solution with plants and the means of DIC content in 2 flasks without plants in the present study) from photosynthesis during the dark period would decrease resulting in reduced utilization of DIC. In other words, the increased utilization of DIC in the dark must result from calcification, as observed in HL-HC (Fig. 2). In our study, the calculation of the utilization of DIC did not take CO_2 produced by dark respiration into consideration. Therefore, the utilization rate of DIC by calcification in darkness was underestimated.

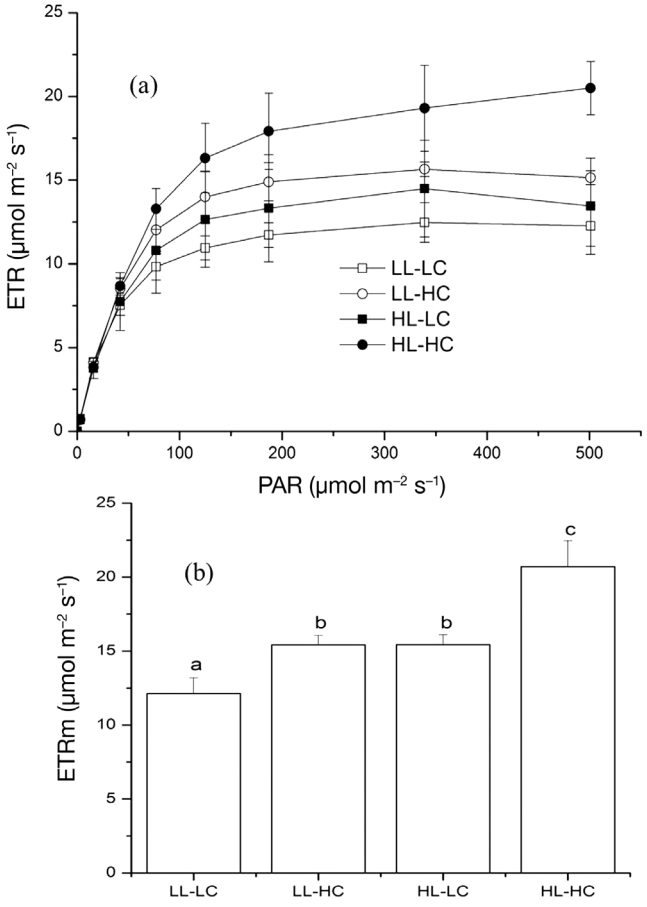

Fig. 5. (a) Electron transport rate (ETR) curves at 2 light levels (LL and HL) and 2 dissolved inorganic carbon (DIC) levels (LC and HC), and (b) maximum relative electron transport rate (rETRm) for these treatments for *Chara vulgaris*. Data are mean ± SD (n = 4). Treatments are defined in Fig. 1

Table 3. *F*-values and significance of 2-way ANOVA of the effects of dissolved inorganic carbon (DIC) and light level on maximum relative electron transport rate (rETRm), photosynthetic rate in light-limited regions (α) and minimum saturating irradiance (Ek) of rapid light curves for *Chara vulgaris* thalli. ***p < 0.001, **p < 0.01, *p < 0.5, ns: p > 0.5

Parameter	DIC (D)	Light (L)	D × L
rETRm	39.330***	23.766***	28.111***
α	0.765 ns	2.266 ns	0.676 ns
Ek	8.849**	14.473***	3.439 ns

Although light is generally not considered to be necessary for calcification, higher calcification rates have been found in the light than in the dark for calcareous plants. This phenomenon is termed light-enhanced calcification and has already been described in some taxonomic groups, including scleractinian corals (e.g. Furla et al. 2000, Al-Horani et al. 2003, 2007), coccolithophorids (e.g. Nimer & Merrett 1992, 1993), and *Chara* spp. (McConnaughey & Falk 1991, Wang 2008). However, in our study, the calcification rate of *Chara* thalli under LL was higher than that under HL (Fig. 3). Light-enhanced calcification occurs through enhanced O_2 production (Rinkevich & Loya 1984) or pH change (Moya et al. 2006). For *Chara* plants, calcification accompanies the photosynthetic utilization of HCO_3^-; therefore, calcification competes with photosynthesis for available DIC. The possible explanation for our finding that the calcification rate is lower under HL might be that, under LC, calcification is limited by the competitive capability of photosynthesis for DIC. This could also explain the higher calcification rate found in HL-HC than in HL-LC.

Dependence of calcification on the utilization of DIC

Here, we identified a linear correlation between calcification rate and the utilization rate of DIC (Fig. 3). Although a similar result has been reported in a coccolithophorid, the level of interaction between photosynthesis and calcification was taxon-dependent (Nimer & Merrett 1992). We reported that a high competition for DIC between photosynthesis and calcification exists in *Chara* thalli (Wang et al. 2013), whereas no such competition was found in *Stylophora pistillata* microcolonies (McConnaughey & Falk 1991, Furla et al. 2000). In the present study, the utilization of DIC resulted from photosynthesis and calcification of plants. We speculate that the high dependence of calcification on the utilization of DIC in *Chara* thalli was primarily related to the ratio of calcification to photosynthesis (C:P). A consequence of this is that any increase or decrease in the C:P ratio by HCO_3^- addition causes a corresponding change in the calcification to DIC utilization ratio (Wang et al. 2013). According to our results, high light intensity and increased HCO_3^- likely affect the ratio of calcification to the utilization of DIC by affecting the C:P ratio.

Owing to the sound understanding of the relationships between fluorescence parameters and photosynthetic electron transport *in vivo* (Maxwell & Johnson 2000, Baker 2008), RLCs of chlorophyll fluorescence can provide direct evidence about the effects of light level and additional HCO_3^- on photosynthesis in *Chara* thalli. In the present study, both light increase and addition of HCO_3^- significantly increased

the ETRm and Ek of RLCs (Fig. 5), indicating that light and external DIC increased the photosynthetic rate and adaptive capacity of the plant to a high light level, which has been reported for some seagrasses (Björk et al. 1997, Schwarz et al. 2000). Our finding of a significant positive interaction on ETRm between light and HCO_3^- levels indicated that the photosynthetic performance of *Chara* thalli was the highest under both HL and HC.

Calcification in *Chara* thalli does not require light (McConnaughey & Whelan 1997). Therefore, light increased the utilization of DIC by photosynthesis, resulting in decreased DIC utilization by calcification. Thus, the ratio of calcification to the utilization of DIC decreased at HL relative to LL (Fig. 4, Table 1). With HCO_3^- addition, both photosynthesis (Fig. 5) and calcification rate increased and resulted in increased utilization of DIC (Fig. 4). However, extant carbon added as HCO_3^- was preferentially incorporated into calcite (Sikes et al. 1980). This suggested a higher C:P ratio, and correspondingly, a higher ratio of calcification to the utilization of DIC for LL-HC and HL-HC treatments (Fig. 4). Results from pH-drift experiments revealed that higher HCO_3^--alkalinity suggested a stronger buffer capability (Kahara & Vermaat 2003). Our finding that there is a significant decrease in the pH of the solution in response to additional HCO_3^- is in line with the findings of the pH-drift experiments. This is also supported by the finding that external HCO_3^- inhibited the increase in solution pH by increasing calcification of calcareous plants, as reported by Wang et al. (2013).

CONCLUSION

Here, we showed that when endogenous inorganic carbon is exhausted, addition of HCO_3^- significantly promoted the calcification rate of *Chara* thalli. Calcification of *C. vulgaris* was primary restrained by HCO_3^- alkalinity in an open 24 h experiment. Furthermore, calcification of *Chara* thalli was dependent on the utilization of DIC; we propose that this dependence is determined by the ratio of calcification to photosynthesis affected by light level and DIC level. Therefore, calcification or $CaCO_3$ encrustations of *Chara* might be a useful indication of water chemistry in the field.

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