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ORIGINAL ARTICLE

The role of microalgae and their carbonic anhydrase on the biological dissolution of limestone

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Abstract This study aims to investigate the effect of microalgae and their carbonic anhydrase (CAex) on limestone dissolution. The dynamics of Ca^{2+} and Mg^{2+} release, the model for the amount of Mg^{2+} released and biological cumulative effect time by the microalgae *Chlamydomonas reinhardtii* (*CR*) and *Chlorella pyrenoedosa* (*CP*), and the algal stable carbon isotopic composition ($\delta^{13}C$) in the presence and absence of the membrane-impermeable CAex inhibitor acetazolamide (AZ) were compared in a medium containing limestone. The amount of Mg^{2+} released from the limestone in the treatment without AZ was more than that with AZ during the logarithmic phase. The amounts of Mg^{2+} release unit algal biomass and unit time in *CR* and *CP* were 3.37×10^{-4} and 2.44×10^{-4} mg/μg days in the treatment without AZ, respectively, and only 1.99×10^{-4} and 2.19×10^{-4} mg/μg days in the treatment with AZ, respectively. The biological dissolution of the algae increased with increasing algal CAex activity. The variation of Ca^{2+} was influenced by reprecipitation, and the algal limestone dissolution cannot be shown distinctly. The CAex of the microalgae may be beneficial for $CaCO_3$ reprecipitation, and the $\delta^{13}C$ values of the algal cells with AZ were lower than those without AZ. Therefore, AZ not only can inhibit limestone dissolution by inhibiting microalgal growth, but also can reduce limestone dissolution by decreasing CAex catalysis. The results suggest the important influence of microalgae and their CAex on the biokarst process.

Keywords Microalgae · Carbonic anhydrase · Biological dissolution · Limestone · Acetazolamide

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Introduction

The total mass of carbon in carbonate rocks is approximately 5×10^{21} mol, which is considered as the largest inorganic carbon pool (Wallmann and Aloisi 2012). The dissolution of carbonate rocks play important roles in global carbon cycling, alkalinity generation, element migration, and transfer of matter among the oceans, the continents and the atmosphere (Yuan 1997; Li et al. 2009). The potential carbon dioxide (CO_2) sink by carbonate rock dissolution is estimated to be 0.41 billion metric tons of carbon/a in the whole world (Liu and Zhao 2000). Limestone is a representative of carbonate rocks. Therefore, research on limestone dissolution can provide insights into the current carbon cycle and evolution of water chemistry.

The dissolution rates of limestone ($CaCO_3 + CO_2 + H_2O \rightarrow Ca^{2+} + 2HCO_3^-$) are determined by three rate-controlling processes: (1) the kinetics of dissolution at the mineral surface; (2) mass transport by diffusion away from this boundary for the dissolved material Ca^{2+}, HCO_3^-, and CO_3^{2-} and towards this boundary for the reactant CO_2; (3) conversion of CO_2 into H^+ and HCO_3^- (Kaufmann and Dreybrodt 2007; Yadav et al. 2008; Wallin and Bjerle 1989).

Living organisms and their specific enzymes have important roles in karst dynamic systems (Kelly et al. 1998; Yuan and Jiang 2000; Lucas 2001). The majority of dissolved inorganic carbon (DIC) in the ocean and karst

lakes is in the form of bicarbonate. Microalgae can utilize the DIC in the water and approximately a half of the DIC uptake observed is attributable to direct HCO_3^- uptake, which will lead to the change of inorganic carbon balance and formation of "Biological pump" (Cassar 2004); such can reduce the HCO_3^- in water and make the atmosphere CO_2 enter into the water to facilitate the dissolution of carbonate. Meanwhile, microalgae can absorb a number of cations (Ca^{2+}, Mg^{2+}, Fe^{2+}, etc.,) in the water (Knauss and Porter 1954), decrease the ion concentration around the minerals and increase the diffusion rate, thus change the dissolution of carbonate.

The slow reaction $CO_2 + H_2O \rightarrow H^+ + HCO_3^-$ plays an important role in the control of the rates of calcite dissolution and precipitation in the system H_2O–CO_2–$CaCO_3$ (Dreybrodt et al. 1996; Liu and Dreybrod 1997). Carbonic anhydrase (CA; EC 4.2.1.1), a zinc-containing metalloenzyme present in mammals, plants, and algae, catalyzes the reversible interconversion between bicarbonate (HCO_3^-) and CO_2 (Badger and Price 1994; Lindskog 1997; Smith and Ferry 2000). External carbonic anhydrase (CAex) can be inhibited by acetazolamide (AZ), a specific membrane-impermeable CA inhibitor. It has been found that the dissolution rate of limestone could increase by about ten times after adding bovine carbonic anhydrase (CA; EC 4.2.1.1) to a karst system (Liu and Dreybrod 1997; Liu 2001), and the crude solution of microbial CA can enhance Ca^{2+} released from limestone (Li et al. 2007). Moreover, some scholars hold the opinion that microorganism-produced CA may be a major factor influencing Ca^{2+} release and leaching in natural karst systems (Li et al. 2005).

The above studies focused on the effect of bacteria, not microalgae and its CAex on limestone dissolution. Most of these experiments were made by simply adding CA and AZ. They usually made the release of Ca^{2+} as the main evidence for limestone dissolution. The fact that CA can change the growth and metabolism of organisms was ignored, usually. Meanwhile, Ca^{2+} is strongly influenced by reprecipitation because of the low solubility of $CaCO_3$. Many biological factors, such as proteins, polypeptides, amino acids and polysaccharides, were found to have potential effects for the formation of $CaCO_3$ crystals (Naka and Chujo 2001; Manoli and Dalas 2001; Meldrum 2003; Hayashi et al. 2008). Microorganism may induce the precipitation of $CaCO_3$ in the process of utilizing the inorganic carbon through CA (Mirjafari et al. 2007; Li et al. 2010). Those will lead to some deviation between the fact and the experiment result that make the release of Ca^{2+} as the main evidence. Due to the higher solubility of $MgCO_3$, Mg^{2+} is almost not affected by reprecipitation. Besides, the content of Mg is only second to that of Ca in the limestone with constant Ca/Mg value in homogeneous rocks. Therefore, this study selected *Chlamydomonas reinhardtii* (*CR*) and *Chlorella pyrenoedosa* (*CP*), both of which are model organism that widely distribute in karst lake (Wu et al. 2008), to study the biological dissolution of limestone. The amount of Mg^{2+} released from the limestone in the medium adding or not AZ, with or without microalgae will be acquired, and the limestone dissolution calculated. Whether microalgae and their CA have important effects on limestone dissolution will be determined, and the driving mechanisms of limestone dissolution by the CAex of microalgae will be clarified.

Materials and methods

Materials and experimental treatments

The limestone was collected from Hongfeng Lake, Guiyang, China. All samples are fresh and free of weathering. Its mole ratio of CaO and MgO was 76.5:1, as determined through high-pressure hydrothermal decomposition (HNO_3:HF = 3:1) and inductively coupled plasma-optical emission spectrometry (ICP–OES, Vista MPX, USA) (Huang and Schulte 1985; Topper and Kotuby-Amacher 1990). A limestone sample was cleaned, air-dried and crushed in a shatter box equipped with a tungsten carbide grinding container. The crushed rock was wet sieved to isolate the 0.2–0.3 mm size fraction. To remove fine particles resulting from the crushing process, sieved samples were repeatedly ultrasonic-washed in ultrapure water and dried at 50 °C.

Chlamydomonas reinhardtii and *Chlorella pyrenoedosa* samples were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. Both species were grown in axenically artificial freshwater soil extract (SE) medium containing 0.25 g/l $NaNO_3$, 0.075 g/l $K_2HPO_4{\cdot}3H_2O$, 0.075 g/l $MgSO_4{\cdot}7H_2O$, 0.025 g/l $CaCl_2{\cdot}2H_2O$, 0.175 g/l KH_2PO_4, 0.025 g/l NaCl, 0.0005 g/l $FeCl_3{\cdot}6H_2O$, 1 ml/l $C_{10}H_{12}N_2O_8FeNa{\cdot}3H_2O$ (0.82 ml/l HCl, 0.1802 g/l $C_{10}H_{14}N_2Na_2O_8{\cdot}2H_2O$ and 0.9010 g/l $FeCl_3{\cdot}6H_2O$), 1 ml/l trace metal solution [2.86 g/l H_3BO_3, 1.86 g/l $MnCl_2{\cdot}4H_2O$, 0.22 g/l $ZnSO_4{\cdot}7H_2O$, 0.39 g/l $Na_2MoO_4{\cdot}2H_2O$, 0.08 g/l $CuSO_4{\cdot}5H_2O$, and 0.05 g/l $Co(NO_3)_2{\cdot}6H_2O$], and 40 ml/l soil extract solution (200 g/l soil). Cultures were incubated at 25.0 ± 1.0 °C under 150 μmol/m^2 s light intensity and a 16/8-h light/dark cycle. Experiments were conducted in 50-ml SE medium-depleted soil extract and with the following treatments (Table 1).

All experimental treatments were carried out in three replicates.

Table 1 Experiment treatments of limestone dissolution

Name	Microalgae	Limestone (g)	Acetazolamide (AZ) (mmol/l)
Treatment 1	*CR* or *CP*	–	–
Treatment 2	*CR* or *CP*	0.5	–
Treatment 3	*CR* or *CP*	0.5	10
Treatment 4	–	0.5	–
Treatment 5	–	0.5	10

CR Chlamydomonas reinhardtii, *CP Chlorella pyrenoedosa*, "–" not addition

Determination of Mg^{2+} and Ca^{2+} concentrations and microalgal biomass

After 1, 3, 5, 7, and 9 days of culture, 5-ml sample aliquots were obtained from the culture medium. The samples were centrifuged, decanted, and filtered (0.22 μm, polyethersulfone). The filtrate was analyzed for the concentrations of Ca^{2+} and Mg^{2+} using ICP–OES (Vista MPX, USA). The pH of the filtrate was also determined using a pH meter (Youke PHS-3C, Shanghai, China). The precipitation of algae was used to measure the concentration of algal chlorophyll-a (chl-a) using a UV spectrophotometer (Labtech UV-2000, Boston, USA) (Huang and Cong 2007).

To eliminate the physical and chemical dissolution effects, the Ca^{2+} and Mg^{2+} concentrations of the solution with the initial Ca^{2+} or Mg^{2+} concentration of the culture medium were calibrated to show the biological dissolution:

$$\mathrm{CT}_i = C_i \cdot C_0/\mathrm{CO}_i \quad (1)$$

where CT_i and C_i (mg/l) are the concentrations of Ca^{2+} or Mg^{2+} in the culture medium under the treatment with both limestone and algae before and after the calibration at the same sampling time, respectively; CO_i (mg/l) is the concentration of Ca^{2+} or Mg^{2+} in the culture medium containing only limestone at the same sampling time; C_0 is the initial concentration of Ca^{2+} or Mg^{2+} in the culture medium; and i is the sampling time.

The Ca^{2+} and Mg^{2+} contents of algae in pure culture were measured by high-pressure hydrothermal decomposition (HNO_3:H_2O_2 = 2:1) and ICP–OES (Vista MPX, USA) (Huang and Schulte 1985; Topper and Kotuby-Amacher 1990).

Amount of Ca^{2+} and Mg^{2+} released

Limestone dissolution was determined by the amounts of Ca^{2+} and Mg^{2+} released from the limestone, i.e., the sum of the change in the amounts of Ca^{2+} and Mg^{2+} in the culture medium and the amounts of Ca^{2+} and Mg^{2+} absorbed by the algae. Therefore, the amount of Ca^{2+} or Mg^{2+} released from the initial culture to the i sampling time in unit volume medium was determined by the following formula:

$$N_i = (\mathrm{CT}_i - C_0) + (\mathrm{Ch}_i - \mathrm{Ch}_0) \cdot C_\mathrm{p}\ (i = 1, 3, 5, 7, 9) \quad (2)$$

where N_i (mg/l) is the amount of Ca^{2+} or Mg^{2+} released from the initial culture to i sampling time in unit volume medium, C_0 is the initial Ca^{2+} or Mg^{2+} concentration of the culture medium, CT_i (mg/l) is the calibrated Ca^{2+} or Mg^{2+} concentration of the culture medium at the i sampling time, Ch_0 and Ch_i (μg/l) are the initial concentrations of algal chl-a in the culture medium and at the i sampling time, respectively; and C_p (mg/μg) is the content of Ca^{2+} or Mg^{2+} corresponding to a unit mass of algal chl-a in pure culture.

Establishment of the model between the dissolution amount with action time in a unit mass of algal chl-a

The equation of the concentration of algal chl-a (Ch_i) in the culture medium with time (T) was established (SigmaPlot 10.0) as $\mathrm{Ch}_i = \mathrm{f}(T)$. The equation of the biological cumulative action time (PT_i) with culture time (T) (days) was then obtained by the integration of the culture time in integral time:

$$\mathrm{PT}_i = \int_0^T \mathrm{Ch}_i \cdot \mathrm{d}(T) \quad (3)$$

The PT_i under different culture times was calculated according to the Eq. (3). The equation between N_i and PT_i was established (SigmaPlot 10.0) as $N_i = \mathrm{f}(\mathrm{PT}_i)$, and the amount of Ca^{2+} or Mg^{2+} released per unit algae based on chl-a content in unit time (P_i) was obtained by the difference between N_i and PT_i, as $P_i = \frac{\mathrm{d}(N_i(\mathrm{PT}_i))}{\mathrm{d}(\mathrm{PT}_i)}$.

Measurements of the ratios of stable carbon isotopic composition ($\delta^{13}C$)

The algal cultures were added with 1 mol/l of hydrochloric acid to wipe off the inorganic carbon and then dried by vacuum freeze-drying prior to the analysis. The cultures were converted to CO_2 at 850 °C in a quartz tube over copper oxide in an oxygen atmosphere. Water and oxygen were removed from the gas stream in a liquid N_2 trap, and CO_2 was double distilled and collected into a sample tube. The CO_2 sample was analyzed with an isotopic ratio mass spectrometer (Finnigan MAT 252, Bremen, Germany). All isotopic compositions ($\delta^{13}C$) were expressed as per mile (‰) and compared with a standard (Pee Dee Belemnite) [Formula (4)]. The analytical precision was ±0.1 ‰.

$$\delta^{13}C(‰) = \left[\left(R_\mathrm{sample}/R_\mathrm{standard}\right) - 1\right] \times 1{,}000 \quad (4)$$

where R_sample and R_standard are the ratios of the heavy to light isotopes ($^{13}C/^{12}C$) of the sample and the standard, respectively.

Results and discussion

Interaction between limestone dissolution and algal growth

Various observations on the correlations between chl-a concentration and algal biomass led to an acceptance of chl-a analysis as an indirect measure of phytoplankton biomass (Vörös and Padisák 1991). The concentration of Chl-a in the culture media with limestone was obviously higher than that without limestone (Fig. 1a). In addition, the microalgae cultured in the medium with limestone for 3–5 days entered the logarithmic growth phase. The microalgae cultured in the medium without limestone shortly entered into the logarithmic phase; after 5–9 days of culture, they maintained in the stationary phase (Fig. 1c). During the entire culture period, the chl-a contents of *CR* and *CP* increased by 60 and 47 times in the treatment with limestone, respectively, and by only 7.7 and 5.3 times in the treatment without limestone, respectively. In addition, the chl-a contents of *CR* and *CP* cultured in the medium with AZ were less than those without AZ. This finding may be attributed to the inhibition of AZ on the inorganic carbon utilization and photosynthesis of microalgae (Beer and Israel 1990).

The algal biomass in the culture medium with limestone for varying culture times can be better fitted by the exponential growth equation (Table 2). The equation showed that limestone dissolution extended the algal logarithmic growth period. However, the algal biomass in the culture medium without limestone for varying culture times can only be fitted by the logistic equation (Table 2). Based on the logistic equation, the largest biomass values of *CR* and *CP* cultured in the medium without limestone were only 1,496 and 1,558 μg/l, respectively. However, the algal biomass in the culture medium with limestone was more than nine times larger than that without limestone. Limestone dissolution can release ions to supply nutrients as the culture medium depleted soil extract. Therefore, limestone dissolution can drive the growth of the microalgae, which utilized rock-dissolved nutrient elements. The logistic equation can fit the relationship between the algal biomass and the culture time. The deficiency in calcium, magnesium, and other nutrient elements limited the growth of algae cultured in the culture medium without limestone.

Inhibition of Mg^{2+} released from limestone by AZ

The part of Ca and Mg released from the limestone was assimilated and utilized by algae, whereas the other was either dissolved in the culture medium or reprecipitated in the form of crystal upon biomineralization (Simkiss and Wilbur 1989). Compared with Ca, Mg is a limiting factor in algal growth. The amount of Ca released increased with increasing calcium carbonate solubility. The amounts of reprecipitated calcium carbonate were observed in the culture medium. The amount of reprecipitated calcium carbonate was difficult to estimate. Therefore, the change in the amount of Mg in the culture medium and the amount of Mg used by the algae indicated the limestone dissolution upon the action of algae.

After 0–3 days of culture, the amount of Mg^{2+} released from the limestone in the treatment without microalgae was greater than that with microalgae (Fig. 2). However, after 5–9 days of culture (logarithmic growth phase), the amount of Mg^{2+} released from the limestone in the treatment with microalgae was the largest (Fig. 2). After 0–3 days of culture, the microalgae were in adjustment, and the main influencing factor was chemical dissolution. However, biological dissolution was dominant when the algae were in the logarithmic growth phase.

After 5–9 days of culture (logarithmic growth phase), the amount of Mg^{2+} released from the limestone in the culture treatment without AZ was greater than that with AZ (Fig. 2). This result indicated that AZ significantly inhibited the biological limestone dissolution by the algae in the logarithmic growth phase. The utilization of Mg and HCO_3^- increased with the growth of the algae, which

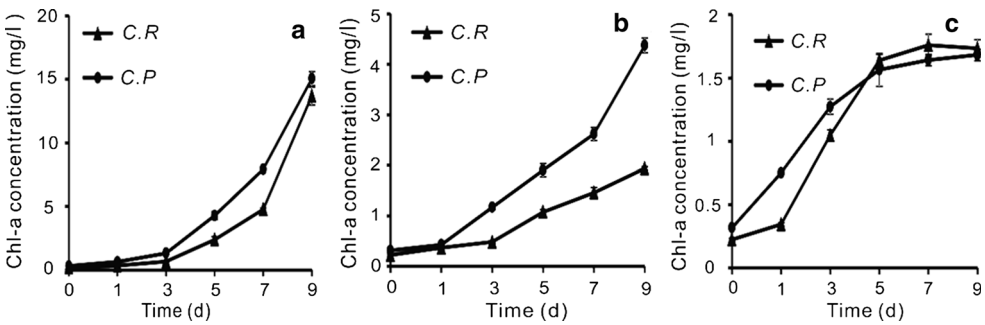

Fig. 1 Variation in the concentration of the Chl-a of *CR* (*filled triangle*) and *CP* (*filled circle*) in the culture media with **a** microalgae and limestone (Treatment 2); **b** microalgae, limestone, and 10 mmol/L AZ (Treatment 3); and **c** microalgae only (Treatment 1)

Table 2 Growth rate and biological cumulative action time model

Sample	Chl-a concentration (Q_i) and time (t) model	Biological cumulative effect time (PT_i) model
CR (−AZ)	$Ch_i = 159 + 139.56e^{0.56T}$ $R^2 = 0.998$ $P = 0.0001$	$PTi = \int_0^T Ch_i d(T) = 159T + 273.65e^{0.51T} - 273.65$
CR (+AZ)	$Ch_i = -1111 + 1313.43e^{0.095T}$ $R^2 = 0.986$ $P = 0.0016$	$PT_i = \int_0^T Ch_i d(T) = -1111T + 13825.57e^{0.095T} - 13825.57$
CP (−AZ)	$Ch_i = -959 + 1138.98e^{0.29T}$ $R^2 = 0.998$ $P < 0.0001$	$PT_i = \int_0^T Ch_i d(T) = -959T + 3927.52e^{0.29T} - 3927.52$
CP (+AZ)	$Ch_i = -723 + 1050.62e^{0.17T}$ $R^2 = 0.993$ $P = 0.0006$	$PT_i = \int_0^T Ch_i d(T) = -723T + 6180.12e^{0.17T} - 6180.12$
CR	$Ch_i = 277 + \frac{1496.65}{1+\left(\frac{T}{-2.95}\right)^{-4.27}}$ $R^2 = 0.997$ $P = 0.0042$	
CP	$Ch_i = 319 + \frac{1558.65}{1+\left(\frac{T}{2.05}\right)^{-1.38}}$ $R^2 = 0.998$ $P = 0.0025$	

CR or *CP*, treatment 1; *CR* (−AZ) or *CP* (−AZ), treatment 2; *CR* (+AZ) or *CP* (+AZ), treatment 3

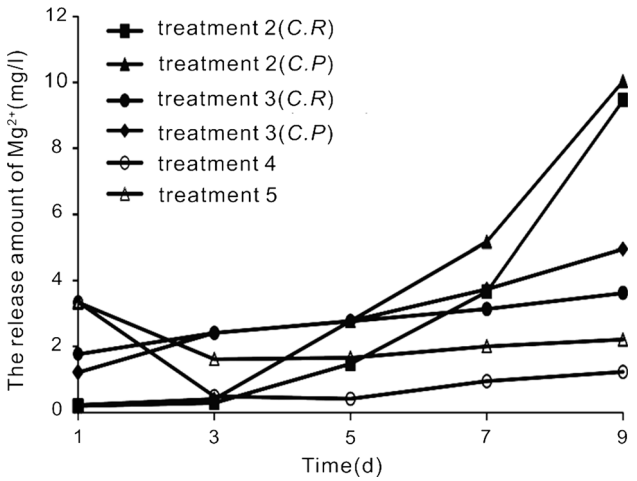

Fig. 2 Amount of Mg^{2+} released from limestone under different treatments with time

constantly moved the equation $CaMg(CO_3)_2 + 2H_2O + 2CO_2 \leftrightarrow Mg^{2+} + Ca^{2+} + 4HCO_3^-$ to the direction of the dissolution. After 1–5 days of culture, rich amounts of Ca^{2+}, Mg^{2+}, and inorganic carbon were found in the medium. The microalgae mainly utilized the elements from the culture medium and hardly used the elements from limestone through biological dissolution. AZ was not inhibited in the biological dissolution of limestone. The microalgae were in the stationary phase from day 5 because of the lack of sufficient nutrient elements. At this time, the biomass of the algae increased slightly as they utilized a large amount of Mg. However, deviations were observed in the content of Mg in the algae under pure cultivation. Such deviation can apparently cause a decreased amount of released Mg^{2+} in the CAex non-inhibited system than in the CAex-inhibited system.

However, whether AZ inhibited the Mg^{2+} released from the limestone either by decreasing the growth or by decreasing the growth and the CAex activity remains unknown. Thus, a model to calculate the amount of Mg^{2+} dissolution per unit algal biomass was established and unit time was based on chl-a content (Table 3). In the system without AZ, the amounts of Mg^{2+} released per unit algal biomass and unit time in *CR* and *CP* were 3.37×10^{-4} and 2.44×10^{-4} mg/μg-chl-d, respectively, whereas those in the system with AZ were 1.99×10^{-4} and 2.19×10^{-4} mg/μg-chl-d, respectively (Table 3). This result showed that AZ can inhibit the Mg^{2+} released from the limestone by decreasing CAex activity.

CAex can catalyze the reversible hydration of CO_2 [$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$]. In the absence of CAex, the balance takes a minute. In the presence of CAex, it only takes 10^{-6} s (Steiner et al. 1975). In the presence of CAex, HCO_3^- was constantly catalyzed and utilized, which shifted the equation $CaMg(CO_3)_2 + 2H_2O + 2CO_2 \leftrightarrow Ca^{2+} + Mg^{2+} + 4HCO_3^-$ to the direction of the dissolution. The activity of CAex in *CR* was higher than that in *CP* (Aizawa and Miyachi 1986). The biological dissolution of limestone by *CR* was stronger that by *CP*, and the inhibition of AZ in *CR* was more obvious than that in *CP*. The biological dissolution by algae strengthened with increasing CAex activity in the algae. The highest activities of

Table 3 Model of the amount of Mg^{2+} released per unit algal biomass and unit time (mg/μg-chl-d)

Sample	Amount of Mg^{2+} released (N_i) and biological cumulative effect time (PT_i) model	Amount of algae dissolution unit algal biomass and unit time (P_i)
CR (−AZ)	$Ni = 0.012 + 3.37 \times 10^{-4}\mathrm{PT_i}$ $R^2 = 0.999$ $P < 0.0001$	$P_i = \frac{d(N_i(\mathrm{PT}_i))}{d(\mathrm{PT}_i)} = 3.37 \times 10^{-4}$
CR (+AZ)	$N_i = 2.01 + 1.99 \times 10^{-4}\mathrm{PT_i}$ $R^2 = 0.916$ $P = 0.0107$	$P_i = \frac{d(N_i(\mathrm{PT}_i))}{d(\mathrm{PT}_i)} = 1.99 \times 10^{-4}$
CP (−AZ)	$N_i = 0.25 + 2.44 \times 10^{-4}\mathrm{PT}_i$ $R^2 = 0.990$ $P = 0.0004$	$P_i = \frac{d(Ni(PT_i))}{d(PT_i)} = 2.44 \times 10^{-4}$
CP (+AZ)	$N_i = 1.61 + 2.19 \times 10^{-4}\mathrm{PT}_i$ $R^2 = 0.945$ $P = 0.0056$	$P_i = \frac{d(N_i(\mathrm{PT}_i))}{d(\mathrm{PT}_i)} = 2.19 \times 10^{-4}$

CR (−AZ) or *CP* (−AZ), treatment 2; *CR* (+AZ) or *CP* (+AZ), treatment 3

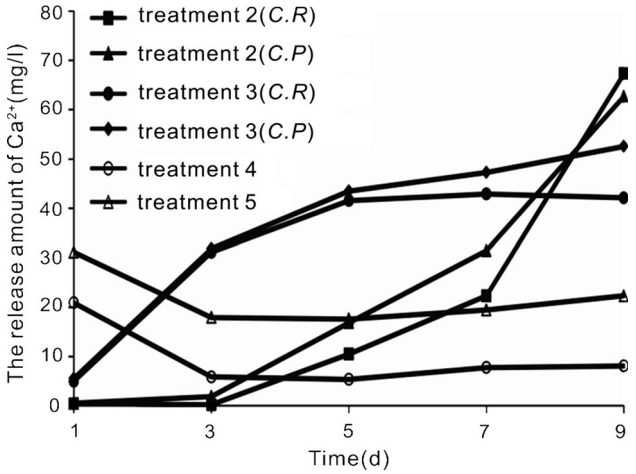

Fig. 3 Amount of Ca^{2+} released from limestone under different treatments with time

marine algae occurred in spring bloom, whereas the lowest values were found in summer with low nutrient environments (Hobson et al. 2002). Therefore, the algal dissolution of limestone was the strongest in spring.

Effect of AZ on Ca^{2+} released from limestone

The state of Ca^{2+} release was more complex because of the influence of many factors, such as chemical precipitation and dissolution, algal assimilation, adsorption, etc.

After the adjustment phase of algal growth (about 0–3 days), the amount of Ca^{2+} released from limestone in the culture treatment with microalgae was greater than that without microalgae (Fig. 3). This result showed that microalgae can promote the Ca^{2+} released from limestone and that the biological dissolution was dominant during the logarithmic growth phase of algal growth (about 5–9 days culture).

The amount of Ca^{2+} released from limestone under the culture treatment with AZ was significantly higher than that without AZ during the 0–7 days culture period (Fig. 3). The Ca^{2+} of the culture medium was supersaturated because of the low solubility of $CaCO_3$ (K_{sp}, $CaCO_3 = 8.7 \times 10^{-9}$, $K = 298.15$). The Ca^{2+} released from limestone would reprecipitate or recrystallize. Meanwhile, along with the utilization of HCO_3^- by microalgae, $CaCO_3$ precipitation would also be produced ($Ca^{2+} + 2HCO_3^- \rightarrow CaCO_3\downarrow + CH_2O + O_2\uparrow$) (McConnaughey 1998). Therefore, AZ accelerated the release of Ca^{2+} from the limestone, in which AZ decreased the utilization of HCO_3^- by the microalgae by decreasing the CAex activity and improved $CaCO_3$ solubility by reducing the pH in the culture medium.

To further prove the effect of reprecipitation, we estimated the concentration of saturated Ca^{2+} under the current experiment conditions. The $CaCO_3$ formation or dissolution was influenced by its ionic solution product:

$$\left[Ca^{2+}\right]\left[CO_3^{2-}\right] = K_{sp}, CaCO_3 = 8.7 \times 10^{-9}(K = 298.15) \quad (5)$$

where K_{sp}, $CaCO_3$ is the $CaCO_3$ solubility product constant. Calcium carbonate forms when the ionic concentration product of Ca^{2+} and CO_3^{2-} was greater than the K_{sp}, $CaCO_3$.

Meanwhile, the concentration of CO_3^- was influenced by pH, HCO_3^- concentration, and the dynamic equilibrium of $[HCO_3^- \leftrightarrow CO_3^{2-} + H^+]$ in the system:

$$K_{a2} = \left[CO_3^{2-}\right][H^+]/\left[HCO_3^-\right] = 10^{-10.33}(K = 298.15) \quad (6)$$

where K_{a2} is the ionization constant of HCO_3^-, $[H^+]$ is the concentration of H^+ (Table 4), and $[HCO_3^-]$ is the concentration of HCO_3^- (Table 4). $[H^+]$ and $[HCO_3^-]$ were obtained by pH measurement and Aquamerck titration, respectively.

Table 4 pH and concentration of HCO_3^- (mmol/l) in the culture medium

Sample	pH						HCO_3^- (9 days)
	0 days	1 day	3 days	5 days	7 days	9 days	
CR (−AZ)	7.26	8.12	8.69	8.78	8.64	8.83	2.43
CP (−AZ)	7.28	8.08	8.63	8.71	8.69	8.81	2.49
CR (+AZ)	7.01	7.31	7.51	7.58	7.62	7.67	4.35
CP (+AZ)	7.02	7.38	7.53	7.50	7.61	7.68	4.35

CR (−AZ) or *CP* (−AZ), treatment 2; *CR* (+AZ) or *CP* (+AZ), treatment 3

After 3–9 days of culture, the concentration of Ca^{2+} and pH in the culture medium was in the equilibrium state. During this culture period, the concentration of HCO_3^- approached that in natural karst water (Wang et al. 2001). Therefore, it can be used to estimate the concentration of saturated Ca^{2+} and the amount of reprecipitated $CaCO_3$ in the culture medium according to Formulas (5) and (6). The results are shown in Tables 5 and 6, respectively. After 3–9 days of culture, the actual Ca^{2+} concentration in the culture medium was higher than the theoretical Ca^{2+} saturated concentration under all treatments. Regardless of the theoretical or actual Ca^{2+} saturated concentration, the value in the culture medium with AZ was larger by an order of magnitude than that without AZ. Saturation and reprecipitation greatly influenced the Ca^{2+} released from the limestone.

$MgCO_3$ cannot reprecipitate in the culture medium under our experiment situation because of its high solubility product constant (K_{sp}, $MgCO_3 = 3.5 \times 10^{-7.46}$). Therefore, given the mass ratio of Ca to Mg (91:1), we can deduce the amount of Ca^{2+} released according to that of Mg^{2+} released from the limestone. The results are shown in Table 6, in which the amount of Ca^{2+} released from the limestone in the culture treatment without AZ was greater than that with AZ when the microalgae were in the middle of the logarithmic phase (5–9 days culture).

Table 5 Theoretical saturated concentration and actual concentration of Ca^{2+} in the culture medium (mmol/l)

Sample	1 day	3 days	5 days	7 days	9 days
CR[a] (−AZ)	0.70	0.19	0.15	0.21	0.14
CR[b] (−AZ)	0.77	0.29	0.33	0.34	0.22
CP[a] (−AZ)	0.62	0.18	0.15	0.15	0.12
CP[b] (−AZ)	0.78	0.30	0.35	0.30	0.22
CR[a] (+AZ)	2.09	1.32	1.12	1.03	0.91
CR[b] (+AZ)	1.57	3.04	3.56	3.75	3.83
CP[a] (+AZ)	1.78	1.26	1.35	1.05	0.89
CP[b] (+AZ)	1.64	2.87	3.47	3.75	3.89

[a] Theoretical saturated concentration

[b] Actual concentration

CR (−AZ) or *CP* (−AZ), treatment 2; *CR* (+AZ) or *CP* (+AZ), treatment 3

Using the estimated value of the amount of Ca^{2+} released (R) minus the measured value of the amount of Ca^{2+} released (M), we obtained the amount of $CaCO_3$ reprecipitated (P) (Table 6). Although deviations were observed in our estimate, they did not influence our qualitative discussion. Compared with the treatment with AZ, the amount of $CaCO_3$ reprecipitated was obviously higher than that without AZ. Some scholars reported that CA can enhance the biomimetic sequestration of CO_2 into $CaCO_3$ (Sharma and Bhattacharya 2010). Therefore, we also suggested that the algal CAex can promote $CaCO_3$ reprecipitation in our experiment. Limestone dissolution and reprecipitation crystallization may occur in karst areas (Buhmann and Dreybrodt 1985). In these processes, Mg, Sr, Ba, Ag, Bi, and other elements of limestone migrate and enrich, whereas Ca reprecipitates and forms a greater amount of pure calcite under appropriate conditions. Given that Ca^{2+} was preferentially removed by calcite precipitation, the ratios of Ca/Mg, Ca/Na, and Ca/Sr in stream water decreased as the degree of supersaturation with respect to calcite increased, and calcite precipitation was estimated to remove up to 70 % of the Ca^{2+} originally derived from carbonate weathering (Jacobson et al. 2002). The bivariate mixture model is usually used to calculate the weathering proportion of silicate and carbonate rock according to the ratios of Ca/Mg, Ca/Na, and Ca/Sr of the chemical composition in river water (Krishnaswami and Singh 1998; Quade et al. 2003). However, the limestone dissolution and $CaCO_3$ reprecipitation induced by aquatic organism led to the great deviation of the ratios of Ca/Mg, Ca/Na, and Ca/Sr in river water. Therefore, special attention should be given to the use of the bivariate mixture model in calculating for the weathering proportion of silicate and carbonate rocks in watersheds containing organisms with high carbonic anhydrase activity.

Stable carbon isotopic evidence of limestone dissolution promoted by microalgaal CAex

Stable carbon isotopic composition ($\delta^{13}C$) has been widely used as a biomarker in studies of matter source and food web (Boschker and Middelburg 2002). In the present study,

Table 6 Estimation values of the amount of Ca^{2+} released and $CaCO_3$ reprecipitated (mg/l)

Sample	1 day		3 days		5 days		7 days		9 days	
	R	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*
CR (−AZ)	18	17	26	26	134	124	333	310	860	793
CP (−AZ)	20	20	37	35	252	235	470	439	913	850
CR (+AZ)	161	156	219	188	252	210	285	243	329	287
CP (+AZ)	111	105	220	188	253	209	340	293	450	398

R, estimated value of the amount of Ca^{2+} released from the limestone (mg/l); *P*, amount of $CaCO_3$ reprecipitated (mg/l); *CR* (−AZ) or *CP* (−AZ), treatment 2; *CR* (+AZ) or *CP* (+AZ), treatment 3

Table 7 Stable carbon isotopic composition ($\delta^{13}C$) of *CR* and *CP*

Sample	$\delta^{13}C_{PDB}$ (‰)	Std.
CP (−AZ)	−21.81	0.12
CP (+AZ)	−29.97	0.20
CR (−AZ)	−22.19	0.22
CR (+AZ)	−27.62	0.30

CR (−AZ) or *CP* (−AZ), treatment 2; *CR* (+AZ) or *CP* (+AZ), treatment 3

we used algal $\delta^{13}C$ to trace the inorganic carbon utilization pathways and the interaction between the microalgae and limestone. The kinetic isotopic fractionation was directly dependent on the reaction rate. The kinetic isotopic fractionation decreased with increasing reaction rate. The uncatalyzed, slow interconversion between CO_2 and HCO_3^- produced approximately 10 ‰ of stable carbon isotopic fractionation, whereas the interconversion in vitro catalyzed by CA only had 1.1 ‰ fractionation (Mook et al. 1974; Marlier and O'Leary 1984). As shown in Table 7, the $\delta^{13}C$ values of microalgae cultured in the medium with AZ were lower than those without AZ. This finding indicated that the kinetic carbon isotopic fractionation produced by the microalgae cultured in the medium without AZ was less than that with AZ. Therefore, we conclude that the HCO_3^- dissolution is accelerated by CAex along with the increase in the utilization of inorganic carbon transformed from HCO_3^- in the aquatic ecosystem of $CaCO_3$–CO_2–H_2O-photoautotroph (Lerman and Mackenzie 2005; Smith and Gattuso 2011).

Conclusions

Limestone dissolution can promote microalgal growth, whereas microalgal growth can also promote limestone dissolution. In the adjustment phase, the chemical dissolution is the main influencing factor. However, when the algae are in the logarithmic growth phase, the biological dissolution is dominant. CAex has an important role in the biological dissolution of limestone. CAex can promote the dissolution of limestone by promoting the growth of microalgae and the reversible hydration of CO_2 [$HCO_3^- + H^+ \leftrightarrow CO_2 + H_2O$] to accelerate the reaction rate of $CaMg(CO_3)_2 + 2H_2O + 2CO_2 \leftrightarrow Ca^{2+} + Mg^{2+} + 4HCO_3^-$ to promote limestone dissolution. In the presence of CAex, the amounts of Mg^{2+} released from limestone per unit algal biomass and unit time in *CR* and *CP* are 3.37×10^{-4} and 2.44×10^{-4} mg/μg days, respectively. In the absence of CAex, the amounts of Mg^{2+} released from limestone per unit algal biomass and unit time in *CR* and *CP* are 1.99×10^{-4} and 2.19×10^{-4} mg/μg days, respectively. The biological dissolution of microalgae increases with increasing CAex activity. Moreover, the CAex of microalgae may promote $CaCO_3$ reprecipitation. Therefore, special attention should be given to the use of the bivariate mixture model in calculating for the proportion of silicate and carbonate rock weathering via the ratios of Ca/Mg, Ca/Na, and Ca/Sr of chemical composition in river water. In conclusion, the algal growth and CAex have important roles in promoting limestone dissolution and reprecipitation.

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