Carbonates Evaporites (2014) 29:203–210
DOI 10.1007/s13146-013-0170-5

ORIGINAL ARTICLE

Preliminary study on the utilization of Ca^{2+} and HCO_3^- in karst water by different sources of *Chlorella vulgaris*

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Accepted: 18 August 2013 / Published online: 11 September 2013

Abstract By choosing exogenous *Chlorella vulgaris* and native *Chlorella vulgaris* which were screened from karst areas as study objects, and making comparison of the utilization of Ca^{2+} and HCO_3^- in typical karst water by *Chlorella vulgaris* of two different origins in a closed system, the relationship between *Chlorella vulgaris* cell numbers and the utilization rate of Ca^{2+} and HCO_3^- and the pH value change are studied. The results show that the native *Chlorella vulgaris* have higher Ca^{2+} and HCO_3^- use ratio than exogenous *Chlorella vulgaris*, while exogenous *Chlorella vulgaris* utilized more Ca^{2+} than native *Chlorella vulgaris*, but utilized the same amount of HCO_3^-. In addition, exogenous *Chlorella vulgaris* can form $CaCO_3$-rich sediment in the form of extracellular crystal, but native *Chlorella vulgaris* cannot. Furthermore, the pH value change in the closed system revealed that both algae utilized the dissolved carbon dioxide as photosynthetic carbon source and made use of HCO_3^-. Exogenous *Chlorella vulgaris* can absorb 26.3 % Ca^{2+} and 29.6 % HCO_3^- of the karst water, and native *Chlorella vulgaris* makes use of 42.1 % Ca^{2+} and 40.6 % HCO_3^-. As a primary producer in the food chain, the two kinds of aquatic algae transform HCO_3^- into organic matter and take them into the ecological system which shows the net carbon sink effect.

Keywords *Chlorella vulgaris* · Ca^{2+} and HCO_3^- utilization · $CaCO_3$ precipitation mechanism · Photosynthesis · Karst ecosystem · Karst carbon sink effect

Introduction

In the global carbon cycle, karst carbon sink effect has been receiving more and more attention (Yuan 1997; Liu and Zhao 2000; Gombert 2002). To make a detailed study of the land and ocean biota function in biochemistry cycling, the International Council of Scientific Unions (ICSU) established the International Geosphere–Biosphere Program (IGBP) since 1983 and the biogeochemistry study of algae was an important component (Liu et al. 2008). When carbonate rocks dissolved, karstification showed carbon sink effect. On the contrary, when carbonate rocks deposited, karstification showed carbon source effect. The following equation explains the effect:

$$CaCO_3 + H_2O + CO_2 \Leftrightarrow Ca^{2+} + 2HCO_3^-. \qquad (1)$$

The equation above shows that in karst areas, the dissolution of the carbonate rocks directly gives rise to the HCO_3^- concentration in water, generally to 3–5 mmol/L; the concentration is several folds of magnitude than non-karst water (Cao et al. 2012). Lerman and Mackenzie (2005) have revealed that hydrophytes abundantly utilize dissolved HCO_3^- as photosynthesis carbon source, at the

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same time generating organic carbon and forming $CaCO_3$ precipitation. The equation is as follows:

$$Ca^{2+} + 2HCO_3^- \rightarrow CaCO_3 \downarrow + CH_2O + O_2 \uparrow . \qquad (2)$$

Hence in karst water environment, the aquatic algae photosynthesis produces net carbon sink effect. In the biogeochemical cycle, algae are an important biological group in both time scale and biomass scale. Moreover, the role of algae is the biggest not only in the biogeochemical cycle of elements, but also in the lithosphere (Wu 1987). Based on the above, we did the following research.

Currently, the related researches of algae focus on the utilization of dissolved inorganic carbon (DIC) and the precipitation of $CaCO_3$ (Zondervan 2007; Sekino and Shiraiwa 1994). Raven (1997) had proved that many marine microalgae could engender mass of carbonic anhydrase and catalyze dissolved HCO_3^- as carbon source. By conducting a pH-drift trial, (Liu et al. 2010) showed that Oocystis solitaria Wittr can make use of dissolved HCO_3^- as inorganic carbon source for photosynthesis and also proved that karst water possesses fertilization effect on its growth. In Lampert's and Sommer's opinion (2008), aquatic algae which have the ability to utilize dissolved HCO_3^- tend to absorb free CO_2 as inorganic carbon source as long as there is adequate CO_2. Yet, which carbon source the algae tends to use is decided by the concentration of dissolved HCO_3^- and CO_2 and its affinity constant $K_{1/2}$. The smaller the constant, the more likely that the algae cell uses dissolved CO_2. In exponential phase cells, dissolved HCO_3^- is the main way of inorganic carbon source utilization and is also related to calcification. In stationary phase cells, dissolved free CO_2 is the main pattern of inorganic carbon source utilization and extracellular carbonic anhydrases exist (Surif and Raven 1989). Both Zaitseva et al. (2006) and Ushatinskaya et al. (2006) had studied the mechanism of $CaCO_3$ deposit under different pH values, illuminations and culture condition of Cyanophyta.

Currently, aquatic algae carbon sink effect is a hot topic in karst studies. In this paper, we studied the utilization of Ca^{2+} and HCO_3^- in karst water by *Chlorella vulgaris* of two different origins, the relationship between algae cell numbers and Ca^{2+}, HCO_3^- utilization and the corresponding change of pH value. The goal was to estimate the dissolved HCO_3^- quantity which was converted by *Chlorella vulgaris* and to compare the karst carbon sink potential of the two different origins of *Chlorella vulgaris*.

Materials and methods

Biological material

Chlorella vulgaris belongs to the *Chlorella*, single-celled algae with a diameter of 3–8 μm in freshwater and one of the earliest lives on earth. It appeared in more than 2 billion years ago without any gene changes since then. *Chlorella vulgaris* is a high-efficiency photosynthetic plant which reproduces in the form of photosynthetic autotrophic and is scattered widely. It can be found in moist soil, rocks and trunks (Hu et al. 1980; Wei 2003).

Exogenous *Chlorella vulgaris* which is called non-karst *Chlorella vulgaris* was obtained from the College of Life Science in South-Central University for Nationalities, situated in central China.

Native *Chlorella vulgaris*, commonly called karst *Chlorella vulgaris*, had been screened in karst moist rocks within typical karst areas.

Cultivation system

The culture medium uses BG-11 which can be referenced from the Freshwater Algae Culture Collection of the Institute of Hydrobiology in Wuhan, China. Karst water was collected from typical karst areas in Guilin Haiyang-Zhaidi subterranean river experimental research site (geographic coordinates: 25°14′11.46″E, 110°33′24.51″N) in Guangxi Province, China. During the configuration of the culture medium, the karst water was used to replace the usual double distilled water. The concentrations of Ca^{2+} and HCO_3^- in the karst water were 76 mg/L and 3.2 mmol/L, respectively. The free CO_2 in the karst water was 0.405 mg, with a pH value of 7.73. A series of 100 ml sealed plastic bottles was filled with 80 mL culture medium with the same quantity algae cells (1.6×10^9 cells) and divided into three groups with eight bottles each. To one group of these bottles exogenous *Chlorella vulgaris* was added and to the other group native *Chlorella vulgaris* was added, while to the third group just culture medium without algae was added and used as blank control. The closed cultivation systems except the blank control consisted of *Chlorella vulgaris*, culture medium and 1/5 (V/V) air. All groups were incubated at 25 ± 1 °C, 2,000 1× for 7 days. Every 24 h, one bottle from each group was separately taken out for measurement of Ca^{2+} and HCO_3^- concentration, free CO_2 content, cell numbers and pH value.

Parameters measurement

Blood counting chamber was used to count the cell numbers in each bottle. WTW340i multifunctional water quality parameters analyzer was used for pH value measurement. Free CO_2 content was titrated with standard NaOH with a concentration 9.704×10^{-3} mol/L. Concentrations of Ca^{2+} and HCO_3^- were measured by Aquamerck alkalinity test and hardness test (Merck Company, German).

Quantification test of $CaCO_3$ deposit

The last bottle of each group was taken out for $CaCO_3$ deposit test. To confirm the quantity of $CaCO_3$ deposit, all the medium solutions of the two bottles were gradually poured out and dried. After that, 2 mL of 0.5 mol/L HCl was added to dissolve the deposit. 2 μL of the dissolved solid was taken out for Ca^{2+} concentration test by atomic absorption spectroscopy (analytikjena ZEEnit700, Jena Company, Germany).

Results and discussions

Comparison of the Ca^{2+} utilization of the two different origins of *Chlorella vulgaris*

Because of the utilization of Ca^{2+} by algae photosynthesis, the Ca^{2+} concentration of the exogenous *Chlorella vulgaris* group decreased from 76 to 42 mg/L. The Ca^{2+} concentration of the native *Chlorella vulgaris* group decreased to 44 mg/L in the Ca^{2+} utilization process. Finally, the Ca^{2+} concentration of the blank control group remained at 76 mg/L with minor fluctuation (Fig. 1). Compared to the native *Chlorella vulgaris* group, the exogenous *Chlorella vulgaris* group had experienced different variation of Ca^{2+} concentration due to the $CaCO_3$ precipitation mechanism. In this study, exogenous *Chlorella vulgaris* can precipitate a portion of dissolved inorganic carbon in the form of $CaCO_3$. The atomic absorption spectroscopy test shows that 0.0281 mmol Ca^{2+} was precipitated in the form of extracellular $CaCO_3$ in exogenous *Chlorella vulgaris* group, but neither the native algae group nor the blank control group showed $CaCO_3$ precipitation. Native *Chlorella vulgaris* transforms more dissolved inorganic carbon engendered by karstification into organic matter than exogenous *Chlorella vulgaris*. After that, all of the organic carbon was cycled into the ecosystem.

According to Berridge et al. (1998), Ca^{2+} acts as an intracellular messenger, triggers life at fertilization and controls the development and differentiation of cells into specialized types. In the cell scale Ca^{2+} controls cell development and death. In both groups, Ca^{2+} concentration generally decreases with the growth of algae cells (Figs. 2, 3), but when Ca^{2+} concentration reaches a constant state, the algae numbers start decreasing acutely. Relational analysis (Figs. 4, 5) reveals that there are very significant negative correlations between algae numbers and Ca^{2+} concentration in the exogenous group and native group. Exogenous *Chlorella vulgaris* has significantly higher negative correlation than native *Chlorella vulgaris*, mainly because of $CaCO_3$ precipitation mechanism in the closed system. In the native *Chlorella vulgaris* group, the Ca^{2+} concentration decreased slightly due to limited resource; but unrestricted cell increase in the closed system finally led to the death of some *Chlorella vulgaris* along with release of intracellular Ca^{2+}. This directly resulted in an increase of Ca^{2+} concentration in the system. But for the exogenous *Chlorella vulgaris* group, the Ca^{2+} concentration experienced a decreasing trend until it reached a constant state that may due to the $CaCO_3$ precipitation mechanism. The $CaCO_3$ precipitation mechanism acts as a regulator in controlling the concentration of Ca^{2+} in the closed system. At the same time, the Ca^{2+} acts as an intracellular messenger control for cell development and death (Merz 1992). Blue algae can emit intracellular Ca^{2+} and absorb extracellular Ca^{2+}. Through this transportation approach, the algae can distinguish different environmental stimuli (Lu 2010).

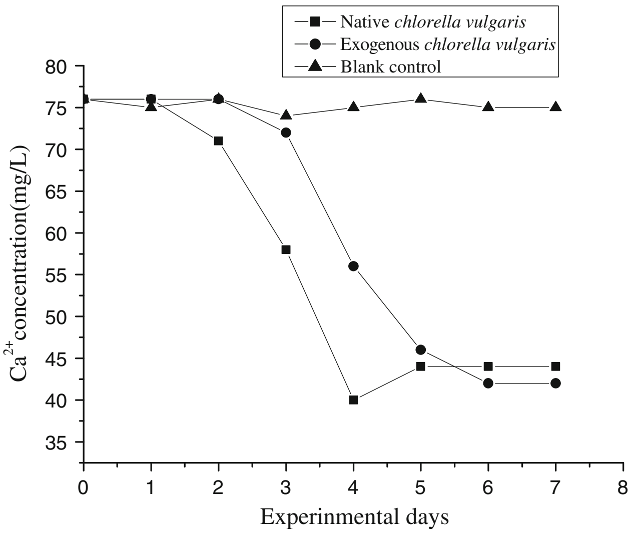


Fig. 1 The alteration curve of Ca^{2+} concentration after separately adding *Chlorella vulgaris* of two different origins to two copies of the same culture medium and the blank control group

Comparison of the HCO_3^- utilization of the two different origin *Chlorella vulgaris*

In the closed system, the dissolved CO_2 decreased from 0.405 to 0 mg consecutively after adding *Chlorella vulgaris* (Fig. 6). On the second day, native *Chlorella vulgaris* appeared with a pH value of 8.97, while the exogenous *Chlorella vulgaris* appeared with a pH value of 8.96 on the third day (Fig. 7). The HCO_3^- concentration increased slightly in the following 2 days in both groups which had been added *Chlorella vulgaris*, and then continued to decrease until constant (Fig. 8). The HCO_3^- concentration in both groups decreased from 3.2 to 1.9 mmol/L, while the total utilization of HCO_3^- was the same. Moreover, the HCO_3^- concentration decreased as the cell numbers

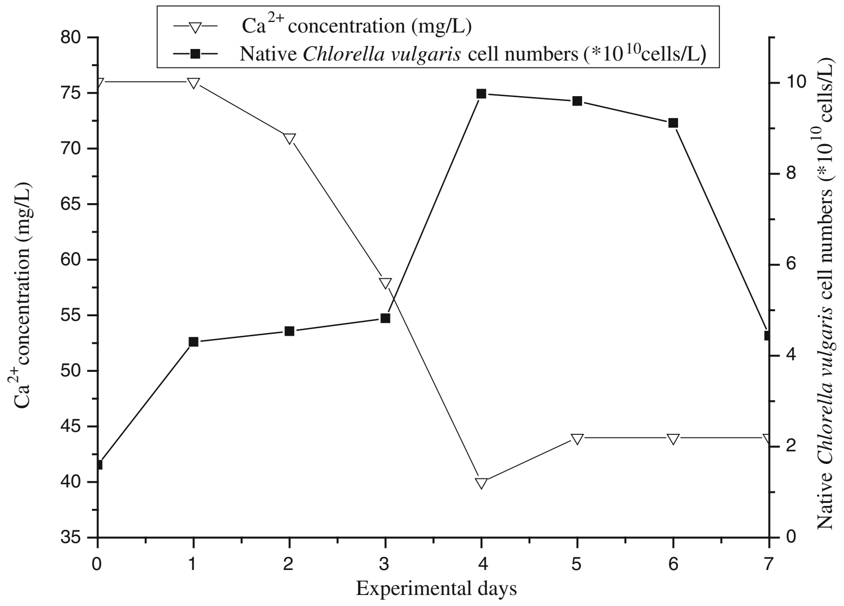


Fig. 2 The relation curves between native *Chlorella vulgaris* cell numbers and Ca^{2+} concentration

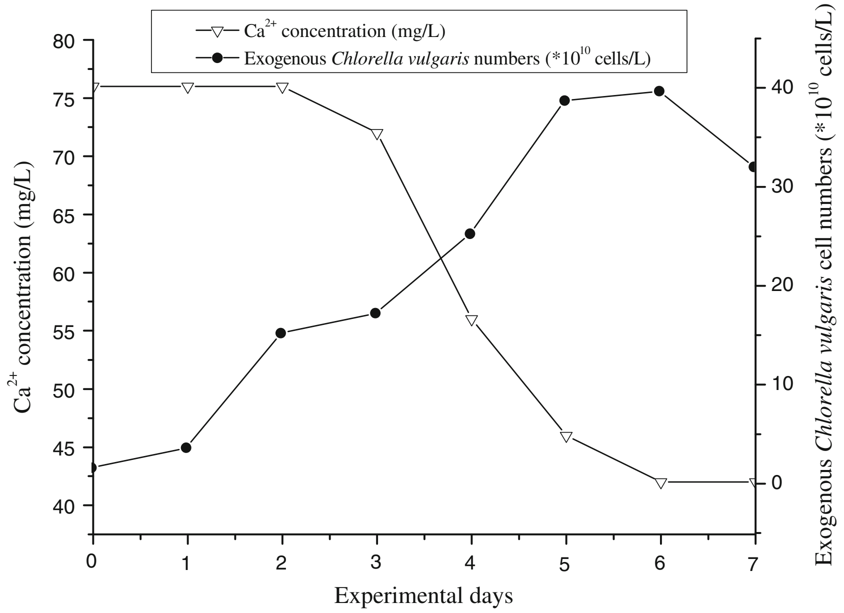


Fig. 3 The relation curves between exogenous *Chlorella vulgaris* cell numbers and Ca^{2+} concentration

increased (Figs. 9, 10). The reason that the HCO_3^- concentration appeared to be slightly increased was mainly due to the carbon source being used by *Chlorella vulgaris* for photosynthesis. When the algae uses inorganic carbon, dissolved CO_2 was firstly utilized (Raven 2003) and then the HCO_3^- (Hellblom and Axelsson 2003) was used as a photosynthetic carbon source (Dong et al. 1993). During the photosynthesis of *Chlorella vulgaris* in the closed system, it can be primarily concluded that *Chlorella vulgaris* firstly utilizes free CO_2 as photosynthetic carbon source and then HCO_3^-.

PH-drift technique is also a universal method in studying inorganic carbon utilization and use capacity (Spence and Maberly 1985). Due to the utilization of inorganic carbon by *Chlorella vulgaris* photosynthesis, pH value in both incubation systems increased from 7.73, respectively, to 10.46 (native *Chlorella vulgaris* group) and 10.52 (exogenous *Chlorella vulgaris* group). Both values were close to a certain stable value which is called pH saturation point (Fig. 7). A pH saturation point around 9 can prove that aquatic algae have the ability to utilize HCO_3^- (Maberly 1990). This implies that not only CO_2,but also HCO_3^- can be a carbon source for *Chlorella vulgaris* photosynthesis. By referring to both Figs. 7 and 8, it is clear that the HCO_3^- concentration in native *Chlorella vulgaris* decreased from the second day when its pH value reached 8.97. However, exogenous *Chlorella vulgaris* started to decrease on the third day when its pH value

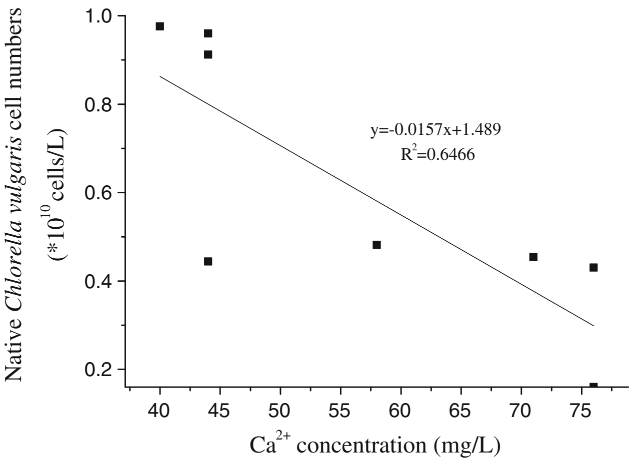


Fig. 4 The relational analysis curve between exogenous *Chlorella vulgaris* cell numbers and Ca^{2+} concentration

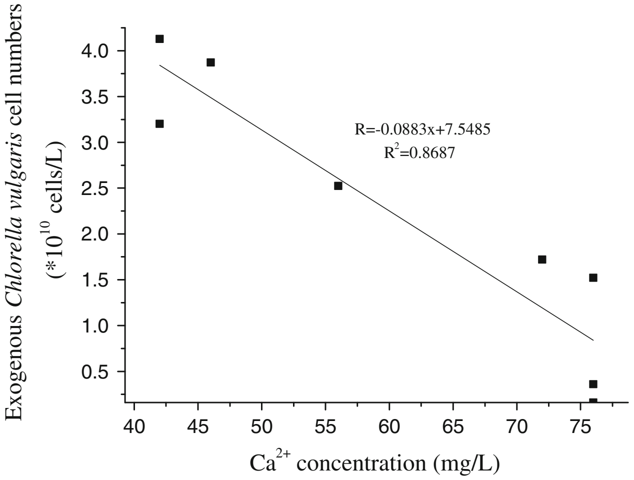


Fig. 5 The relational analysis curve between native *Chlorella vulgaris* cell numbers and Ca^{2+} concentration

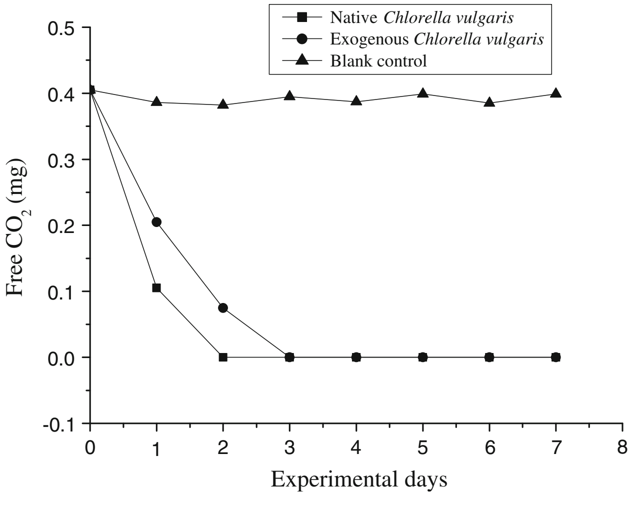


Fig. 6 The change curve of free CO_2 in the three groups of closed system

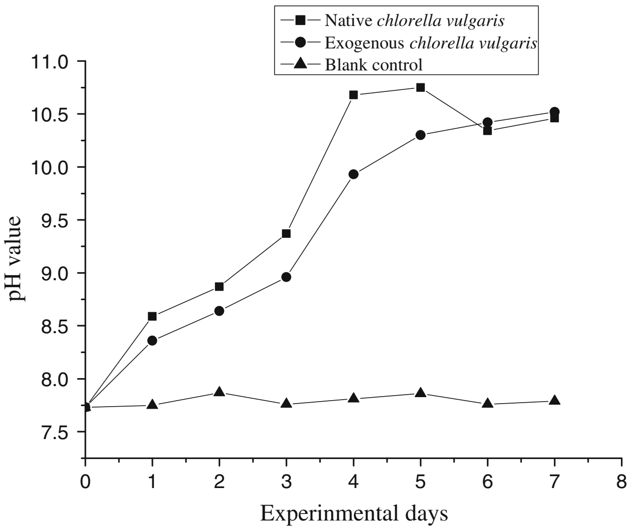


Fig. 7 The alteration curve of pH concentration after separately adding *Chlorella vulgaris* of two different origins to two copies of the same culture medium and the blank control group

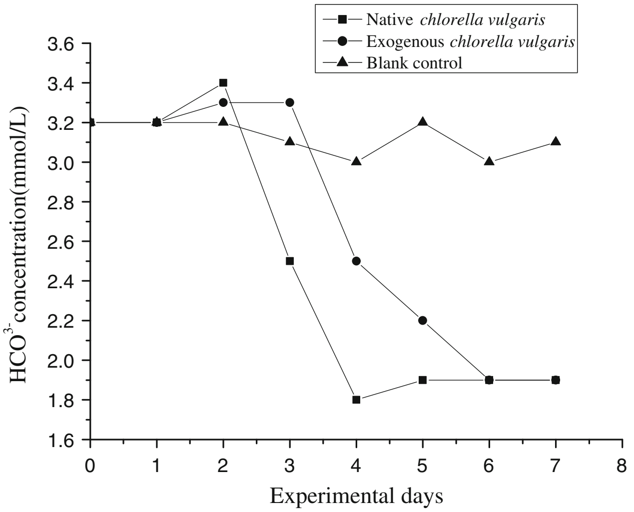


Fig. 8 The alteration curve of HCO_3^- concentration after separately adding *Chlorella vulgaris* of two different origins to two copies of the same culture medium and the blank control group

reached 8.96. The result verifies that due to the photosynthesis of *Chlorella vulgaris*, there are no dissolved CO_2 exist in the water environment when the pH value reaches around 9. The result also proves that during the inorganic carbon utilization, dissolved CO_2 will be used first, and then HCO_3^- will be used after CO_2 is used up.

Karst carbon sink transformation quantity by *Chlorella vulgaris* of two different origins

In the cultivation system, the gross Ca^{2+} and HCO_3^- were 0.152 and 0.256 mmol, respectively. Due to the photosynthesis of *Chlorella vulgaris*, the net decrements of Ca^{2+}

Fig. 9 The relation curves between HCO_3^- concentration and native *Chlorella vulgaris* cell numbers

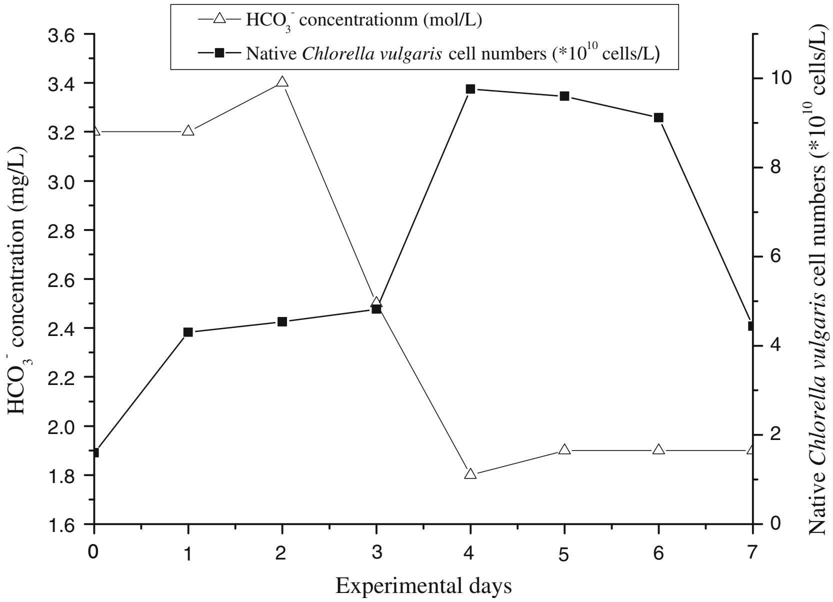
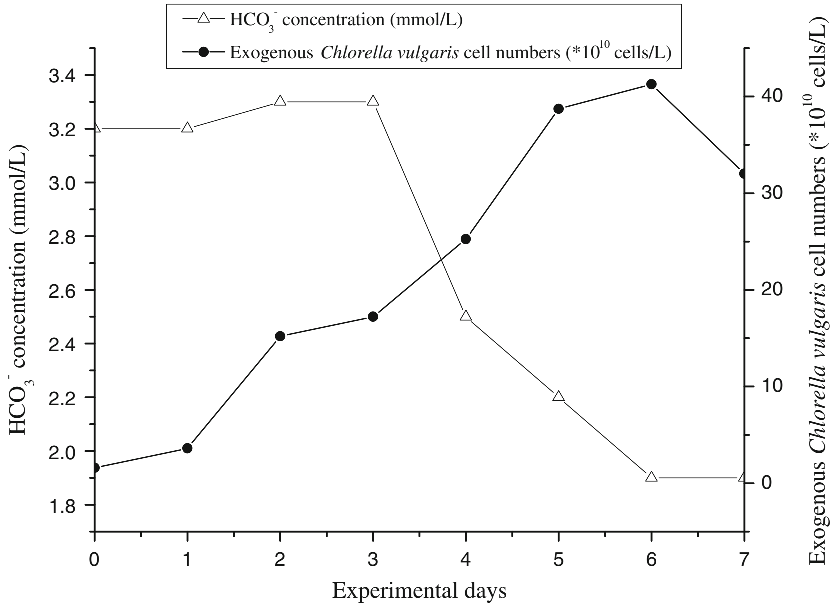


Fig. 10 The relation curves between HCO_3^- concentration and exogenous *Chlorella vulgaris* cell numbers



and HCO_3^- quantity in native *Chlorella vulgaris* were 0.064 and 0.104 mmol, respectively. In exogenous *Chlorella vulgaris*, the net decrement Ca^{2+} and HCO_3^- quantities were 0.068 and 0.104 mmol, respectively. By utilizing the HCO_3^- as a carbon source to photosynthesis, the inorganic carbon which originated from karst carbon sink was converted to organic matters in the form of biomass. According to McConnaughey (1991), some algae can generate $CaCO_3$ crystals on the surface of their cells when using HCO_3^- as carbon source in photosynthesis. In exogenous *Chlorella vulgaris*, accompanied by 0.0281 mmol $CaCO_3$ precipitation an equal amount of HCO_3^- which supplied carbon for $CaCO_3$ were consumed, so by deducting 0.0281 mmol there are 0.0759 mmol HCO_3^- transformed into organic matter, which accounts for 29.6 % of the gross HCO_3^- in the closed system had been transformed into organic matter, but to native *Chlorella vulgaris* the amount account for 40.6 %. Table 1 shows the results of HCO_3^- consumption by these two kinds of *Chlorella vulgaris*. By absorbing the Ca^{2+} as intracellular messenger to control the growth of *Chlorella vulgaris*, native *Chlorella vulgaris* utilized all of the deduced Ca^{2+}. However, in exogenous *Chlorella vulgaris* a part of the deduced Ca^{2+} was used to generate $CaCO_3$ precipitation, which accounts for 18.5 %. Table 2 shows the results of Ca^{2+} consumption by these two kinds of *Chlorella vulgaris*. According to Downing et al. (1993), in the total carbon in all lakes around the world, about 69.1 %

Table 1 HCO_3^- consumption by two different origins of *Chlorella vulgaris*

Chlorella vulgaris origins	Net reduction (mmol)	Organic matter (mmol)	$CaCO_3$ precipitation (mmol)	Residual (mmol)
Native *Chlorella vulgaris*	0.104	0.104	0	0.152
Exogenous *Chlorella vulgaris*	0.104	0.0759	0.0281	0.152

Net reduction means the total HCO_3^- decrement after cultivating *Chlorella vulgaris* for 7 days in the closed system. Organic matter stands for the inorganic HCO_3^- quantity which transformed into organic material by *Chlorella vulgaris*

Table 2 Ca^{2+} consumption by two different origins of *Chlorella vulgaris*

Chlorella vulgaris origins	Net reduction (mmol)	Absorbed by *Chlorella vulgaris* (mmol)	$CaCO_3$ precipitation (mmol)	Residual (mmol)
Native *Chlorella vulgaris*	0.064	0.064	0	0.088
Exogenous *Chlorella vulgaris*	0.068	0.0399	0.0281	0.084

Net reduction means the total HCO_3^- decrement after cultivating *Chlorella vulgaris* for 7 days in the closed system. Absorbed by *Chlorella vulgaris* stands for the utilizing amount of Ca^{2+} for its growth

comes from the atmosphere. The remaining 30.9 % comes from other places. In this research, it can be concluded that in the karst water system, about 40.6 % total carbon sink comes from the dissolved carbonate and silicate rocks. Therefore in karst aquatic ecological system, the potential carbon sink of aquatic algae is tremendous and cannot be ignored.

Chlorella vulgaris' karst carbon sink effect

Both origins of *Chlorella vulgaris* show carbon sink effect, and in the closed system the *Chlorella vulgaris* carbon sink capacity was limited by cell numbers. But in the karst aquatic ecological system, the special environment in which water contains abundant Ca^{2+} and HCO_3^- greatly contributes to *Chlorella vulgaris'* carbon sink. For any organism, the main influences for its growth are environmental and ecological factors (Yang 1993). In this study, ecological factors such as illumination, temperature, water resource and so on were suitable for algae growth. Therefore, *Chlorella vulgaris*' growth is restricted by the environmental resources, namely HCO_3^-, which is the photosynthesis carbon source, and Ca^{2+}, which controls the growth and death of *Chlorella vulgaris* cells. Thus, in the closed system the *Chlorella vulgaris* population shows a logistic growth due to a lack of Ca^{2+} and HCO_3^-. However, they are adequate in karst aquatic ecological system. Moreover, ecological factors such as illumination, temperature, water resource and so on are limiting factors. The vast majority of karst in China distribute in the southwest where sunlight, temperature and rainfall are suitable for aquatic algae's growth. Therefore, in karst areas algae photosynthesis greatly contributes to karst carbon sink.

Conclusions

1. In the process of utilizing Ca^{2+}, exogenous *Chlorella vulgaris* uses more Ca^{2+} than native *Chlorella vulgaris*. Both kinds of *Chlorella vulgaris* cell numbers show negative correlation relationship with Ca^{2+} concentration. In the exogenous *Chlorella vulgaris* group, there is extracellular $CaCO_3$ crystal. By comparing with the native *Chlorella vulgaris* group, $CaCO_3$ precipitation mechanism regulates the Ca^{2+} concentration, thus controlling its growth in high Ca^{2+} concentration environment.
2. Both kinds of *Chlorella vulgaris* first utilized dissolved CO_2 as carbon source for photosynthesis and then of HCO_3^-. During the photosynthesis, when pH value is lower than 9, both *Chlorella vulgaris* mainly utilize CO_2 as carbon source. In contrast, when the pH value is higher than 9, HCO_3^- is the photosynthesis carbon source for both *Chlorella vulgaris*.
3. Native *Chlorella vulgaris* can make use of 40.6 % HCO_3^- in the cultivate system, while the exogenous chlorella used 29.6 %. The native *Chlorella vulgaris* possesses more karst carbon sink capacity than exogenous *Chlorella vulgaris*. In karst aquatic ecological system, the aquatic algae's karst carbon sink effects are tremendous.

Acknowledgments We wish to express our sincere gratitude to Yik Yu Au for his help with the manuscript. We also sincerely thank the two anonymous reviewers. This work was supported by the National Natural Science Foundation of China (Grant No. 40872213), the Chinese Geological Survey Projects (No. 121237121302401) and the Special Fund for Public Benefit Scientific Research of Ministry of Land and Resources of China (No. 2002371210001).

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