Effect of acetazolamide on stable carbon isotope fractionation in *Chlamydomonas reinhardtii* and *Chlorella vulgaris*

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The effect of extracellular carbonic anhydrase (CAex) on stable carbon isotope fractionation in algae is still unclear. The stable carbon isotope composition and algal growth in the presence and absence of the membrane-impermeable CA inhibitor acetazolamide were compared in *Chlamydomonas reinhardtii* and *Chlorella vulgaris*. The CAex of both algal species contributed about 9‰ of the stable carbon isotope fractionation and exhibited a dosage effect. Therefore, evidence in vivo that CAex leads to a larger carbon isotope fractionation of algae is presented.

acetazolamide, algae, bicarbonate, carbonic anhydrase, stable carbon isotope

The isotopic composition of sedimented algal material is an indicator of paleoenvironmental conditions because of the well-documented effect of CO2 concentration on marine algal carbon fractionation [1–3]. However, use of existing models for prediction of algal carbon isotope fractionation revealed a large deviation in some aquatic ecosystems [4]. This deviation, if not considered in prediction models, would affect the precision of predictions of paleoenvironmental CO2 concentrations based on the carbon isotope composition.

The deviation from the model-predicted isotope fractionation results not only from environmental factors, but also from some important physiological factors. Extracellular carbonic anhydrase action might be one of the main physiological processes that lead to the deviation. Carbonic anhydrase (CA; EC 4.2.1.1), a zinc-containing metalloenzyme, catalyzes the reversible interconversion between bicarbonate (HCO3−) and CO2. The uncatalyzed, slow interconversion between CO2 and HCO3− produced about 10‰ of the stable carbon isotope fractionation, whereas the interconversion *in vitro* catalyzed by CA had only 1.1‰ fractionation [5,6].

The present study examines the effects of extracellular CA (CAex) on carbon isotope fractionation by comparison of the stable carbon isotope composition and algal growth with and without the membrane-impermeable CA inhibitor acetazolamide (AZ) in *Chlamydomonas reinhardtii* and *Chlorella vulgaris*. Evidence in vivo showed that extracellular CA leads to higher algal carbon isotope fractionation.

1 Materials and methods

*Chlamydomonas reinhardtii* and *Chlorella vulgaris* samples were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. Both species were grown axenically in artificial freshwater soil extract (SE) medium. Cultures were incubated at 25.0 ± 1.0°C under 150 μmol m−2 s−1 light intensity and a 16/8 h day/night cycle. Experiments were conducted with the following treatments.

Treatment 1: *C. reinhardtii* and *C. vulgaris* were grown in SE media that contained different NaHCO3 concentra-
tions (0, 0.5, 2, 8, 16, or 20 mmol L\(^{-1}\)) with or without AZ (10 mmol L\(^{-1}\)). The cultures were treated for 12 d, with the first 8 d for expanding the culture, and the last 4 d for strengthening the culture. The added NaHCO\(_3\), which has a \(\delta^{13}C\) value of \(-17.4\%\) in solid state, and \(-16.6\%\) in solution, was a tracer for the dissolved inorganic carbon (DIC) sources used by algae.

Treatment 2: \(C.\) reinhardtii and \(C.\) vulgaris were grown in SE media with different AZ concentrations (0, 0.01, 0.10, 0.50, or 1.00 mmol L\(^{-1}\)) and 0.5 mmol L\(^{-1}\) added NaHCO\(_3\). The cultures were treated for 14 d, with the first 10 d for expanding the culture, and the last 4 d for strengthening the culture. The added NaHCO\(_3\) was the same \(\delta^{13}C\) value as that of Treatment 1.

All experimental treatments consisted of five replicates. Algal proteins were assayed using Coomassie brilliant blue. Aquamerck was used in the titration of bicarbonate concentrations in the media.

The algal cultures were dried prior to analysis and were converted to CO\(_2\) at 800°C in a quartz tube over copper oxide. 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 isotope ratio mass spectrometer (Finnigan MAT 252, Bremen, Germany). All isotopic compositions (\(\delta^{13}C\)) are expressed as per mille (‰) and compared with a standard (Pee Dee Belemnite) (see Formula (1)). The analytical precision was ±0.1‰.

\[
\delta^{13}C (\%) = [(R_{\text{sample}}/R_{\text{standard}})-1] \times 1000, \tag{1}
\]

where \(R_{\text{sample}}\) and \(R_{\text{standard}}\) are the ratio of heavy to light isotopes (\(^{13}C/^{12}C\)) of the sample and the standard, respectively.

### 2 Results and discussion

#### 2.1 Stable carbon isotope composition with and without acetazolamide

The CO\(_2\) and generated HCO\(_3\) concentrations varied with the amount of added HCO\(_3\). The total DIC content increased with increasing added HCO\(_3\) concentration (Table 1). The \(\delta^{13}C_{\text{DIC}}\) and the proportion of HCO\(_3\) obtained from conversion of CO\(_2\) to total DIC were high at low added HCO\(_3\) concentrations (0–2.00 mmol L\(^{-1}\)), and low at high added HCO\(_3\) concentrations (8.00–20.00 mmol L\(^{-1}\)) regardless of the presence or absence of AZ (Table 1). The pH in the presence of AZ was lower than that without AZ. Therefore, the CO\(_2\) concentrations in the culture media that contained

### Table 1 Concentration of dissolved inorganic carbon and \(\delta^{13}C_{\text{DIC}}\) in original culture media of \(C.\) reinhardtii and \(C.\) vulgaris

| Treatment                  | pH  | [HCO\(_3\)]\(^{(a)}\) (mmol L\(^{-1}\)) | [HCO\(_3\)]\(^{(b)}\) (mmol L\(^{-1}\)) | [HCO\(_3\)]\(^{(c)}\) (mmol L\(^{-1}\)) | [CO\(_2\)]\(^{(d)}\) (mmol L\(^{-1}\)) | Total DIC (mmol L\(^{-1}\)) | \(\delta^{13}C_{\text{DIC}}\) (‰, PDB) |
|----------------------------|-----|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|-------------------------------|---------------------------------|
| **Chlamydomonas reinhardtii** |     |                                       |                                       |                                       |                                       |                               |                                 |
| −AZ                        | 6.50| 0                                    | 0.80                                 | 0.80                                 | 1.41                                 | −11.8                         |
|                           | 6.87| 0.50                                 | 1.60                                 | 1.10                                 | 0.52                                 | 2.12                          |
|                           | 7.75| 2.00                                 | 2.90                                 | 0.90                                 | 0.12                                 | 3.02                          |
|                           | 8.54| 8.00                                 | 8.20                                 | 0.20                                 | 0.06                                 | 8.26                          |
|                           | 8.82| 16.00                                | 15.70                                | −0.30                                | 0.06                                 | 15.76                         |
|                           | 8.88| 20.00                                | 19.40                                | −0.60                                | 0.06                                 | 19.46                         |
| **Chlorella vulgaris**     |     |                                       |                                       |                                       |                                       |                               |                                 |
| −AZ                       | 6.50| 0                                    | 0.80                                 | 0.80                                 | 1.61                                 | −11.8                         |
|                           | 6.87| 0.50                                 | 1.45                                 | 0.95                                 | 0.47                                 | 1.92                          |
|                           | 7.75| 2.00                                 | 2.70                                 | 0.70                                 | 0.11                                 | 2.81                          |
|                           | 8.54| 8.00                                 | 8.20                                 | 0.20                                 | 0.06                                 | 8.26                          |
|                           | 8.82| 16.00                                | 15.45                                | −0.55                                | 0.06                                 | 15.51                         |
|                           | 8.88| 20.00                                | 19.5                                 | −0.50                                | 0.06                                 | 19.56                         |
| **Chlamydomonas**          |     |                                       |                                       |                                       |                                       |                               |                                 |
| **reinhardtii** +AZ        | 6.00| 0                                    | 1.00                                 | 1.00                                 | 2.40                                 | 3.40                          |
|                           | 6.40| 0.50                                 | 1.80                                 | 1.30                                 | 1.72                                 | 3.52                          |
|                           | 7.00| 2.00                                 | 2.90                                 | 0.90                                 | 0.70                                 | 3.60                          |
|                           | 7.62| 8.00                                 | 8.40                                 | 0.40                                 | 0.48                                 | 8.88                          |
|                           | 7.90| 16.00                                | 15.90                                | −0.10                                | 0.48                                 | 16.38                         |
|                           | 8.00| 20.00                                | 19.50                                | −0.50                                | 0.47                                 | 19.97                         |
| **Chlorella vulgaris**     |     |                                       |                                       |                                       |                                       |                               |                                 |
| **+AZ**                   | 6.00| 0                                    | 0.80                                 | 0.80                                 | 1.92                                 | 2.72                          |
|                           | 6.40| 0.50                                 | 1.60                                 | 1.10                                 | 1.53                                 | 3.13                          |
|                           | 7.00| 2.00                                 | 2.80                                 | 0.80                                 | 0.67                                 | 3.47                          |
|                           | 7.62| 8.00                                 | 8.25                                 | 0.25                                 | 0.47                                 | 8.72                          |
|                           | 7.90| 16.00                                | 15.70                                | −0.30                                | 0.47                                 | 16.17                         |
|                           | 8.00| 20.00                                | 19.50                                | −0.50                                | 0.47                                 | 19.97                         |

\(a\) NaHCO\(_3\) concentration added to the culture medium; \(b\) NaHCO\(_3\) concentration in the culture medium; \(c\) HCO\(_3\) concentration from the conversion of CO\(_2\); \(d\) concentration of CO\(_2\) in the medium.
AZ were higher than in the media lacking AZ.

The stable carbon isotope composition and growth of *C. reinhardtii* and *C. vulgaris* varied with the total DIC with and without AZ (Figure 1). Protein content increased at low added HCO$_3^-$ concentrations and decreased at high added HCO$_3^-$ concentrations without AZ. However, in the presence of AZ, the protein content increased independent of HCO$_3^-$ concentration.

The stable carbon isotope composition without AZ was significantly different from that with AZ. The mean $\delta^{13}C$ values of *C. reinhardtii* and *C. vulgaris* without AZ were about 9.1‰ and 11.4‰ more positively skewed than that with AZ at low added HCO$_3^-$ concentrations (0–2.00 mmol L$^{-1}$), respectively. The $\delta^{13}C$ values of the two algal species were similar (about $-25.5‰$) at 8.00 mmol L$^{-1}$ added HCO$_3^-$ regardless of the presence or absence of AZ. The $\delta^{13}C$ values of *C. reinhardtii* and *C. vulgaris* without AZ were more negatively skewed than that with AZ (16.00 and 20.00 mmol L$^{-1}$ added HCO$_3^-$, respectively).

The stable carbon isotope composition in algae reflects the utilization of DIC [7]. Without AZ, the algal cells mainly utilized the HCO$_3^-$ generated from the rapid interconversion of CO$_2$ catalyzed by CAex at low added HCO$_3^-$ concentrations (0–2.00 mmol L$^{-1}$). The carbon isotope fractionation was very low (about 1.1‰) [6]. However, in the presence of AZ, the algal cells mainly used the HCO$_3^-$ generated from the slow (uncatalyzed) interconversion of CO$_2$ at low added HCO$_3^-$ concentrations. The slow interconversion between CO$_2$ and HCO$_3^-$ produced about 10‰ of the stable carbon isotope fractionation [5]. Therefore, $\delta^{13}C$ values in the presence of AZ at low added HCO$_3^-$ concentrations were about 9‰ less than those in the absence of AZ, regardless of algal growth rate or cell size [8].

Algal growth rate and cell size may affect isotope fractionation [8]. The $\delta^{13}C$ value is inversely correlated with algal growth rate or cell size [8]. The difference in the $\delta^{13}C$ values of *C. reinhardtii* with and without AZ was approximately 9‰. This value could be regarded the absolute contribution of CAex. However, greater differences in the $\delta^{13}C$ values than 9‰ (mean 11.4‰) were observed in *C. vulgaris*. A small, additional difference (mean 2.4‰) in the $\delta^{13}C$ values was recorded in *C. vulgaris* above that produced by CAex. The additional difference might reflect the lower algal growth rate and smaller cell size when *C. vulgaris* was cultured in media that contained AZ.

A linear or stoichiometric relationship existed between the $\delta^{13}C$ values in the algal cells and the total DIC in culture media lacking AZ at low added HCO$_3^-$ concentrations. In natural water bodies, the concentration of HCO$_3^-$ is much lower than 2.0 mmol L$^{-1}$ [9]. Thus, we also deduced that the difference in carbon isotopic fractionation is large between algae with high CAex activity and that without CAex activity.

The $\delta^{13}C$ value of added HCO$_3^-$ was more negative than that of the control (0 mmol L$^{-1}$ added HCO$_3^-$) (Table 1). At high concentrations of added HCO$_3^-$ (8.00–20.00 mmol L$^{-1}$), the algal cells mainly used the added HCO$_3^-$ in the presence or absence of AZ. Therefore, the more negative the algal $\delta^{13}C$ values, the more the algal cells used the added HCO$_3^-$ regardless of the growth restrictions. In medium lacking AZ,
growth of the two algal species was inhibited by the DIC sources because of the high pH of the culture media. The HCO$_3^-$ concentration was too high for algal growth in these conditions. The algal cells prioritized uptake of the light $^{12}$C isotope, which resulted in a negatively skewed $\delta^{13}$C value and higher carbon isotope fractionation. Furthermore, carbon isotope fractionation increased with increasing pH and HCO$_3^-$ concentration in the medium. Thus, a linear relationship was observed between the $\delta^{13}$C values in algal cells and the total DIC in culture medium lacking AZ at high added HCO$_3^-$ concentrations (8.00–20.00 mmol L$^{-1}$).

Growth of the two algal species was not inhibited by the DIC sources at high added HCO$_3^-$ concentrations (8.00–20.00 mmol L$^{-1}$) in the presence of AZ because of the moderate pH of the culture medium. The algal cells produced little carbon isotope fractionation during the growth period because of the unrestricted DIC. Therefore, the $\delta^{13}$C values of the algal cells with AZ were higher than those in medium lacking AZ.

The algal growth rate and cell size in C. reinhardtii were higher than those of C. vulgaris at 16.00 and 20.00 mmol L$^{-1}$ added HCO$_3^-$. Therefore, the $\delta^{13}$C values of C. reinhardtii were lower than those of C. vulgaris at 16.00 and 20.00 mmol L$^{-1}$ added HCO$_3^-$. The two algal species showed positively skewed $\delta^{13}$C values because of the slight inhibition of CAex activity by AZ at low concentrations, and negatively skewed $\delta^{13}$C values because of the high inhibition of CAex activity by high AZ concentrations. These results indicated AZ has a dose-dependent effect on algal stable carbon isotope fractionation.

### 2.2 Dosage effect of acetazolamide on the stable carbon isotope signature

Low AZ concentrations (0–0.1 mmol L$^{-1}$) promoted growth of C. reinhardtii, whereas high AZ concentrations (0.5–1 mmol L$^{-1}$) slightly inhibited growth. In the concentration range tested, AZ exhibited no significant effect on the growth of C. vulgaris. The effect of AZ on the stable carbon isotope fractionation of C. reinhardtii was similar to that of C vulgaris (Figure 2). The two algal species showed positively skewed $\delta^{13}$C values because of the slight inhibition of CAex activity by AZ at low concentrations, and negatively skewed $\delta^{13}$C values because of the high inhibition of CAex activity by high AZ concentrations. These results indicated AZ has a dose-dependent effect on algal stable carbon isotope fractionation.

### 3 Conclusions

Extracellular carbonic anhydrase can significantly influence stable carbon isotope fractionation of C. reinhardtii and C. vulgaris under normal growth conditions. The CAex of C. reinhardtii and C. vulgaris contributes approximately 9%o of the stable carbon isotope fractionation, which was derived from the difference between the smaller fractionation from the catalyzed conversion of CO$_2$ to HCO$_3^-$ and the larger fractionation from the uncatalyzed, slow interconversion. Moreover, CAex had a dose-dependent effect on algal stable carbon isotope fractionation, which could cause a large deviation in the predicted paleoenvironmental CO$_2$ concentration based on the algal carbon isotope composition.

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