

## RESEARCH PAPER

# Cellular inorganic carbon fluxes in *Trichodesmium*: a combined approach using measurements and modelling

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## Abstract

To predict effects of climate change on phytoplankton, it is crucial to understand how their mechanisms for carbon acquisition respond to environmental conditions. Aiming to shed light on the responses of extra- and intracellular inorganic C ( $C_i$ ) fluxes, the cyanobacterium *Trichodesmium erythraeum* IMS101 was grown with different nitrogen sources ( $N_2$  vs  $NO_3^-$ ) and  $pCO_2$  levels (380 vs 1400  $\mu atm$ ). Cellular  $C_i$  fluxes were assessed by combining membrane inlet mass spectrometry (MIMS),  $^{13}C$  fractionation measurements, and modelling. Aside from a significant decrease in  $C_i$  affinity at elevated  $pCO_2$  and changes in  $CO_2$  efflux with different N sources, extracellular  $C_i$  fluxes estimated by MIMS were largely unaffected by the treatments.  $^{13}C$  fractionation during biomass production, however, increased with  $pCO_2$ , irrespective of the N source. Strong discrepancies were observed in  $CO_2$  leakage estimates obtained by MIMS and a  $^{13}C$ -based approach, which further increased under elevated  $pCO_2$ . These offsets could be explained by applying a model that comprises extracellular  $CO_2$  and  $HCO_3^-$  fluxes as well as internal  $C_i$  cycling around the carboxysome via the  $CO_2$  uptake facilitator NDH-1<sub>4</sub>. Assuming unidirectional, kinetic fractionation between  $CO_2$  and  $HCO_3^-$  in the cytosol or enzymatic fractionation by NDH-1<sub>4</sub>, both significantly improved the comparability of leakage estimates. Our results highlight the importance of internal  $C_i$  cycling for  $^{13}C$  composition as well as cellular energy budgets of *Trichodesmium*, which ought to be considered in process studies on climate change effects.

**Key words:** Carbon acquisition, carbon-concentrating mechanism (CCM),  $CO_2$ , cyanobacteria, leakage, NDH, ocean acidification.

## Introduction

Cyanobacteria are ancient organisms responsible for oxygenation of the atmosphere during times when  $CO_2$  concentrations were about two orders of magnitude higher than today (cf. Buick, 1992; Kasting and Siefert, 2002). Possibly due to their origin at that time, the  $CO_2$ -fixing enzyme RubisCO of cyanobacteria has one of the lowest affinities among all

autotrophic organisms (Badger *et al.*, 1998; Tortell, 2000). Consequently, cyanobacteria are dependent on high activities of carbon-concentrating mechanisms (CCM) for increasing the  $CO_2$  concentration in the vicinity of RubisCO. Currently, due to ongoing anthropogenic  $CO_2$  combustion, the availability and speciation of inorganic C ( $C_i$ ) in seawater is changing

Abbreviations:  $a_{carb}$ , fractional contribution of  $HCO_3^-$  to total  $C_i$  uptake into the carboxysome;  $a_{cyl}$ , fractional contribution of  $HCO_3^-$  to total  $C_i$  uptake into the cytosol; CA, carbonic anhydrase; CCM, carbon-concentrating mechanism; chl *a*, chlorophyll *a*;  $C_i$ , inorganic carbon; DIC, dissolved inorganic carbon;  $K_{1/2}$ , half-saturation concentration;  $L_{13C}$ , leakage calculated from  $^{13}C$  fractionation;  $L_{carb}$ , modelled leakage from the carboxysome;  $L_{cyl}$ , modelled leakage over the plasma membrane;  $L_{MIMS}$ , leakage estimated by MIMS; MIMS, membrane inlet mass spectrometry; POC, particulate organic carbon; PQ, photosynthetic quotient;  $V_{max}$ , maximal rate;  $\epsilon_{cyl}$ ,  $^{13}C$  fractionation in the cytosol;  $\epsilon_{db}$ ,  $^{13}C$  equilibrium fractionation in the external medium,  $\epsilon_p$ , total  $^{13}C$  fractionation during POC formation;  $\epsilon_{Rub}$ ,  $^{13}C$  fractionation by RubisCO.

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at a rapid pace (IPCC, 2007). In view of this ocean acidification (Caldeira and Wickett, 2003), a number of studies in recent years have focused on the mechanisms of C acquisition and CO<sub>2</sub> responses of different groups of phytoplankton (e.g. Rost *et al.*, 2008). Among these studies, the abundant N<sub>2</sub>-fixing cyanobacterium *Trichodesmium* stands out by showing an exceptionally high stimulation of biomass production and N<sub>2</sub> fixation in response to elevated *p*CO<sub>2</sub> (e.g. Hutchins *et al.*, 2007; Levitan *et al.*, 2007; Kranz *et al.*, 2009). Further studies on the underlying reasons for these CO<sub>2</sub> effects show a decrease in C<sub>i</sub> affinity at high *p*CO<sub>2</sub> (Kranz *et al.*, 2009; Kranz *et al.*, 2010). Given the high energy demand of the CCM in cyanobacteria, a reallocation of energy between C<sub>i</sub> acquisition and N<sub>2</sub> fixation was suggested to stimulate production at high *p*CO<sub>2</sub> (Kranz *et al.*, 2010).

Cellular C<sub>i</sub> affinities of *Trichodesmium* are determined by the interplay of several transporters and structural adaptations composing the CCM. In order to understand *p*CO<sub>2</sub> responses of the CCM as well as potential changes in energy demand, it is necessary to distinguish between these different components. While CO<sub>2</sub> can diffuse through the cell membrane without energy investments, the low equilibrium concentrations, slow interconversion with HCO<sub>3</sub><sup>-</sup> (Zeebe and Wolf-Gladrow, 2007), and its tendency to leak out of the cell compromise the use of CO<sub>2</sub> as the predominant C<sub>i</sub> source. Therefore, cyanobacteria have evolved energy-dependent transporters for taking up HCO<sub>3</sub><sup>-</sup>, which can be accumulated in the cell more efficiently (Badger *et al.*, 2006). *Trichodesmium* has been found to cover ~90% of its C demand using HCO<sub>3</sub><sup>-</sup> (Kranz *et al.*, 2009; Kranz *et al.*, 2010). Uptake of HCO<sub>3</sub><sup>-</sup> in this species is catalysed by the Na<sup>+</sup>-dependent transporter BicA, which is fuelled by Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> symport or via an H<sup>+</sup>/Na<sup>+</sup> antiport mechanism (Price *et al.*, 2008).

Cyanobacterial RubisCO is localized in distinct compartments within the cell, the so-called carboxysomes. The protein shells of these microbodies are permeable to HCO<sub>3</sub><sup>-</sup> but pose a diffusion barrier for CO<sub>2</sub> (Dou *et al.*, 2008; Espie and Kimber, 2011), allowing significant accumulation of CO<sub>2</sub> in the vicinity of RubisCO. Inside the carboxysomes, transformation of HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub> is accelerated by carbonic anhydrase (CA; reviewed by Espie and Kimber, 2011). In addition to direct HCO<sub>3</sub><sup>-</sup> uptake and CO<sub>2</sub> diffusion, CO<sub>2</sub> uptake in *Trichodesmium* is facilitated by the NDH-1<sub>4</sub> complex, which converts CO<sub>2</sub> to HCO<sub>3</sub><sup>-</sup> in the cytoplasm, presumably in a CA-like reaction (Price *et al.*, 2002). The protein complex is thought to be located on the thylakoid membrane and form part of the photosynthetic/respiratory electron transport chain, being fuelled by electrons donated from NADPH or ferredoxin, which are subsequently transferred to the plastoquinone pool (Price *et al.*, 2002). After the hydration of CO<sub>2</sub>, a proton is thought to be released into the thylakoid lumen, contributing to the pH gradient necessary for ATP synthesis and making the reaction irreversible in the light (Price *et al.*, 2002).

Conversion of CO<sub>2</sub> to HCO<sub>3</sub><sup>-</sup> by the NDH complex has been proposed to drive internal C<sub>i</sub> recycling to minimize loss via CO<sub>2</sub> efflux (Maeda *et al.*, 2002; Price *et al.*, 2002). Due to the strong CO<sub>2</sub> accumulation required in cyanobacteria, CO<sub>2</sub>

efflux is a major challenge in these organisms. Despite the interplay of the carboxysome and proposed recapture of CO<sub>2</sub> by the NDH-1<sub>4</sub> complex, efflux of CO<sub>2</sub> has been shown to equal ~50–90% of gross C<sub>i</sub> uptake in *Trichodesmium* (Kranz *et al.*, 2009; Kranz *et al.*, 2010). Next to the C source (CO<sub>2</sub> vs HCO<sub>3</sub><sup>-</sup>), leakage (i.e. CO<sub>2</sub> efflux : gross C<sub>i</sub> uptake) can strongly affect isotopic composition of organic C produced during photosynthesis (Burkhardt *et al.*, 1999; Sharkey and Berry, 1985), and thus measurements of <sup>13</sup>C fractionation can provide complementary information on this aspect of CCM regulation (e.g. Laws *et al.*, 1997; Keller and Morel, 1999; Rost *et al.*, 2006; Tchernov and Lipschultz, 2008). In fact, differences in leakage estimates based on membrane inlet mass spectrometry (MIMS; Badger *et al.*, 1994) and C isotope fractionation (Sharkey and Berry, 1985) have been attributed to internal C<sub>i</sub> cycling driven by NDH (Kranz *et al.*, 2010).

In a previous study (Eichner *et al.*, 2014), the energy allocation to different physiological processes in *Trichodesmium* under varying energetic states was addressed by altering the cellular energy budget through addition of different nitrogen sources: while N<sub>2</sub> fixation is a highly energy-demanding process with a high demand for ATP, NO<sub>3</sub><sup>-</sup> requires very little ATP (only for uptake) but instead has a high electron demand. The study highlighted the dependence of energy reallocation on the stoichiometry in energy demands (ATP vs NADPH) of the different pathways involved. The energy demand of the CCM in *Trichodesmium* remains uncertain, however, especially because the regulation of internal C<sub>i</sub> fluxes is as yet poorly characterized. To shed light on the extra- and intracellular C<sub>i</sub> fluxes under the different energetic conditions, *Trichodesmium* was grown with different *p*CO<sub>2</sub> levels and N sources (N<sub>2</sub> vs NO<sub>3</sub><sup>-</sup>), and a combination of different methods, including MIMS and <sup>13</sup>C fractionation measurements, as well as modelling, was employed. While MIMS provides a useful tool to investigate C<sub>i</sub> fluxes across the cell membrane, internal fluxes cannot be directly measured and were therefore modelled. Model calculations of internal C<sub>i</sub> fluxes made use of the measured extracellular C<sub>i</sub> fluxes and the isotopic composition of particulate organic C (δ<sup>13</sup>C<sub>POC</sub>), which reflects the integrated effects of extra- and intracellular C<sub>i</sub> fluxes. Hereby, a common model of <sup>13</sup>C fractionation (Sharkey and Berry, 1985) was extended by including internal fluxes around the carboxysome.

## Materials and methods

### Culture conditions

*Trichodesmium erythraeum* IMS101 was grown in semi-continuous batch cultures at 25°C and 150 μmol photons m<sup>-2</sup> s<sup>-1</sup> with a 12 h : 12 h light : dark cycle. Cultures were grown in 0.2-μm-filtered artificial seawater (YBCII medium; Chen *et al.*, 1996) and kept in exponential growth phase by regular dilution with culture medium. Culture bottles were continuously bubbled with 0.2-μm-filtered air with *p*CO<sub>2</sub> levels of 380 and 1400 μatm. Prior to experiments, cells were allowed to acclimate to the respective *p*CO<sub>2</sub> for at least 2 weeks. Cultures in which pH had drifted by >0.09 units compared to cell-free reference media were excluded from further analysis. In treatments with NO<sub>3</sub><sup>-</sup> as the N source, 0.2-μm-filtered NaNO<sub>3</sub> was added to achieve mean concentrations of 97 ± 2 μmol l<sup>-1</sup> in the experiments, and these never

fell below 65 μmol l<sup>-1</sup>. Cultures were acclimated to NO<sub>3</sub><sup>-</sup> for at least 1 week before measurements. Samples for the analysis of dissolved inorganic C (DIC) were filtered through 0.2 μm filters and measured colourimetrically (QuAAtro autoanalyzer, Seal, Norderstedt, Germany). Average precision was ±5 μmol kg<sup>-1</sup>. The pH values of the acclimation media were measured potentiometrically (pH meter pH3110, WTW, Weilheim, Germany). For further details on culture conditions as well as carbonate chemistry parameters, see [Eichner et al. \(2014\)](#).

#### MIMS measurements

Cellular C<sub>i</sub> fluxes ([Fig. 1](#)) were obtained using a custom-made MIMS system ([Rost et al., 2007](#)), applying a disequilibrium approach described by [Badger et al. \(1994\)](#). Assays were performed in YBCII medium buffered with HEPES (50 mM, pH 8.0) at acclimation temperature and light intensity, unless otherwise specified. To account for the diurnal cycle of C<sub>i</sub> fluxes in *Trichodesmium*, measurements were performed three times over the day, during time intervals from 0 to 1.5, 5.5 to 7, and 9 to 10.5 h after beginning of the photoperiod. CO<sub>2</sub> and O<sub>2</sub> fluxes were measured as a function of DIC, starting with concentrations close to zero (media bubbled with CO<sub>2</sub>-free air), which were subsequently increased by step-wise addition of NaHCO<sub>3</sub> up to concentrations of ~5000 μM. As the assay medium is buffered, unlike the conditions during acclimation of the cells, the HCO<sub>3</sub><sup>-</sup>:CO<sub>2</sub> ratio stayed constant over the investigated DIC range. DIC-saturated rates of photosynthesis (V<sub>max</sub>) and half-saturation concentrations [K<sub>1/2</sub> (DIC)] were obtained by fitting a Michaelis-Menten function to the data. Net O<sub>2</sub> evolution was converted to C fixation (F<sub>fix</sub>) assuming a photosynthetic quotient (PQ) of 1.34 ([Williams and Robertson, 1991](#)). Net CO<sub>2</sub> uptake (F<sub>cyt, netCO2</sub>) was calculated from the steady-state rate of CO<sub>2</sub> depletion at the end of the light period and corrected for the CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> interconversion

in the medium (F<sub>ext, db</sub>). Using C fixation and net CO<sub>2</sub> uptake, HCO<sub>3</sub><sup>-</sup> uptake rates (F<sub>cyt, HCO3-</sub>) could be derived by a mass balance equation:

$$F_{cyt, HCO_3^-} = F_{fix} - F_{cyt, netCO_2} \quad (1)$$

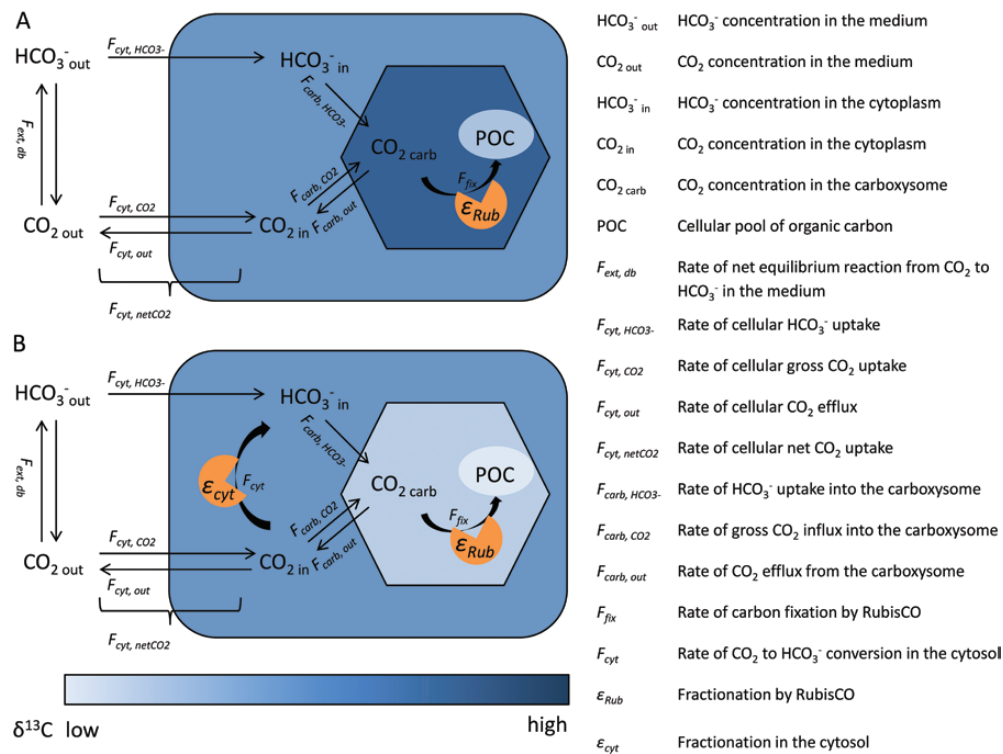
For normalization of the CO<sub>2</sub> and O<sub>2</sub> traces, duplicate samples for chlorophyll *a* (chl *a*) analysis were taken after each measurement. Chl *a* was extracted in acetone for >12 h and determined fluorometrically (TD-700 fluorometer, Turner Designs, Sunnyvale, CA, USA; [Holm-Hansen and Rieman, 1978](#)).

#### Leakage estimation

Cellular leakage was estimated by two different methods. Firstly, leakage was determined by MIMS measurements using the disequilibrium approach ([Badger et al., 1994](#)). Cellular leakage (L<sub>MIMS</sub>) is defined as the ratio of CO<sub>2</sub> efflux (F<sub>cyt, out</sub>) to gross C<sub>i</sub> uptake [i.e. the sum of HCO<sub>3</sub><sup>-</sup> (F<sub>cyt, HCO3-</sub>) and gross CO<sub>2</sub> uptake (F<sub>cyt, CO2</sub>):

$$L_{MIMS} = \frac{F_{cyt, out}}{F_{cyt, HCO_3^-} + F_{cyt, CO_2}} \quad (2)$$

F<sub>cyt, out</sub> was estimated from the initial increase in CO<sub>2</sub> concentration after switching off the light ([Badger et al., 1994](#)). These estimates are based on the assumption that the rate of diffusive CO<sub>2</sub> efflux during the light phase is well represented by the rate of CO<sub>2</sub> efflux during the first ~20 s of the subsequent dark phase. As leakage calculated by this approach is based on O<sub>2</sub> measurements that are converted to C fluxes, the sensitivity to different PQ values was tested by varying PQ between 1.0 and 1.7, yielding deviations of not more than 15% of leakage estimates (i.e. 0.06 units).



**Fig. 1.** Schematic diagram showing the cellular C<sub>i</sub> pools and fluxes characterized by measurements and modelling. Fluxes and concentrations in the external medium and fluxes over the cell membrane as well as C fixation (F<sub>fix</sub>) were measured by MIMS, while fluxes in and out of the carboxysome were modelled. Shading intensity denotes δ<sup>13</sup>C values of different cellular C pools (including POC and C<sub>i</sub> in the cytosol and carboxysome). (A) Fractionation during C fixation by RubisCO leads to depletion of POC in <sup>13</sup>C and enrichment of <sup>13</sup>C in the carboxysomal C<sub>i</sub> pool. (B) Fractionation during internal C<sub>i</sub> cycling, e.g. via NDH, leads to <sup>13</sup>C depletion of the carboxysomal C<sub>i</sub> pool. Consequently, the POC formed is isotopically lighter than in scenario A. This figure is available in colour at [JXB online](#).



In the second approach, leakage was estimated from the isotopic fractionation during POC formation ( $\epsilon_p$ ), which was calculated from the difference in isotopic composition between POC ( $\delta^{13}\text{C}_{\text{POC}}$ ) and  $\text{CO}_2$  ( $\delta^{13}\text{C}_{\text{CO}_2}$ ) in the medium according to Freeman and Hayes (1992). Duplicate samples for analysis of  $\delta^{13}\text{C}_{\text{POC}}$  were filtered onto pre-combusted GF/F filters and acidified with 200  $\mu\text{l}$  HCl (0.2 M) to remove all  $\text{C}_i$  prior to analysis.  $\delta^{13}\text{C}_{\text{POC}}$  was measured with an EA mass spectrometer (ANCA SL 2020, SerCon Ltd, Crewe, UK). For analysis of the isotopic composition of DIC ( $\delta^{13}\text{C}_{\text{DIC}}$ ), filtered samples were fixed with  $\text{HgCl}_2$  (final concentration 110 mg  $\text{l}^{-1}$ ). Subsequent to acidification of the samples, isotopic composition of  $\text{CO}_2$  in the headspace was analysed with an isotope ratio mass spectrometer (GasBench-II coupled to Delta-V advantage, Thermo, Bremen, Germany). The isotopic composition of  $\text{CO}_2$  was calculated from  $\delta^{13}\text{C}_{\text{DIC}}$ , following a mass balance equation (Zeebe and Wolf-Gladrow, 2007). Leakage ( $L_{13\text{C}}$ ) was subsequently derived using an extended equation from Sharkey and Berry (1985):

$$L_{13\text{C}} = \frac{\epsilon_p - (a_{\text{cvt}}\epsilon_{\text{db}})}{\epsilon_{\text{Rub}}} \quad (3)$$

where  $\epsilon_{\text{Rub}}$  is the intrinsic discrimination of  $^{13}\text{C}$  by RubisCO (assumed to be +25‰; Roeske and O'Leary, 1984; Guy et al., 1993) and  $\epsilon_{\text{db}}$  represents the equilibrium fractionation between  $\text{CO}_2$  and  $\text{HCO}_3^-$  (-9‰; Mook et al., 1974). The fractional contribution of  $\text{HCO}_3^-$  to gross  $\text{C}_i$  uptake ( $a_{\text{cvt}}$ ), being introduced by Burkhardt et al. (1999), has been determined by MIMS measurements for the respective treatments. These calculations assume an equilibrium situation and further consider the cell as a single compartment.

## Results and discussion

### General CCM characteristics

MIMS measurements showed a highly efficient CCM with a high capacity for regulation of  $\text{C}_i$  affinity over the diurnal cycle as well as with different  $p\text{CO}_2$  levels, in agreement with previous studies on *Trichodesmium* (e.g. Kranz et al., 2009; Kranz et al., 2010). Half-saturation DIC concentrations for C fixation ( $K_{1/2}$ ) ranged between ~20 and 500  $\mu\text{mol DIC l}^{-1}$  (Supplementary Figure S1), which is equivalent to ~0.2 and 4  $\mu\text{mol CO}_2 \text{ l}^{-1}$  and is thus substantially lower than the  $K_M$  of cyanobacterial RubisCO (105–185  $\mu\text{mol CO}_2 \text{ l}^{-1}$ ; Badger et al., 1998). Taking the ratio of  $K_M$  to  $K_{1/2}$  as a measure of  $\text{CO}_2$  accumulation in the vicinity of RubisCO (assuming

a  $K_M$  of 150  $\mu\text{mol CO}_2 \text{ l}^{-1}$ ), our data suggest accumulation factors between ~35 and 900 and indicate that the degree of RubisCO saturation is always larger than 80%. Accordingly, under the applied external  $\text{CO}_2$  concentrations, concentrations in the carboxysome typically exceed 600  $\mu\text{mol CO}_2 \text{ l}^{-1}$ . The CCM was primarily based on active  $\text{HCO}_3^-$  uptake, accounting for  $82 \pm 4\%$  of gross  $\text{C}_i$  uptake (Table 1). As gross  $\text{C}_i$  uptake was approximately twice as high as net C fixation at acclimation DIC (~2100  $\mu\text{mol CO}_2 \text{ l}^{-1}$ ), leakage measured by MIMS ranged between 0.3 and 0.7 (i.e.  $\text{CO}_2$  efflux equalled 30–70% of gross  $\text{C}_i$  uptake; Table 1). As a consequence of the high  $\text{HCO}_3^-$  contribution and the high  $\text{CO}_2$  efflux, the net fluxes of  $\text{CO}_2$  were generally directed out of the cell (cf. negative values for net  $\text{CO}_2$  uptake: Table 1, Fig. 2).

### Diurnal changes in $\text{C}_i$ fluxes

The diurnal cycle was characterized by low  $K_{1/2}$  values in the morning and a downregulation of C fixation rates at midday (ANOVA,  $P < 0.001$ ; Supplementary Figure S1A and B). Leakage estimated by MIMS at acclimation DIC was lowest in the morning, increased towards midday, and decreased again towards the evening (ANOVA,  $P < 0.05$ ; Table 1). Leakage estimates for DIC levels approaching zero (obtained by curve fits of leakage plotted over DIC concentration; Fig. 2) varied even more over the course of the day, yielding values around 0.3 in the mornings, while at midday and in the evening ratios approached 1.0 (data not shown). These diurnal changes in leakage could be explained by the concurrent changes in the ratio of  $\text{HCO}_3^-$  to  $\text{CO}_2$  uptake (Table 1, Supplementary Figure 1C), which were characterized by low  $\text{CO}_2$  fluxes in the mornings (ANOVA,  $P < 0.05$ ; Table 1), while  $\text{HCO}_3^-$  uptake was higher in the morning than at midday, and increased again towards the evening (ANOVA,  $P < 0.05$ ; Table 1). Over the day, a higher share of  $\text{HCO}_3^-$  uptake, which is less prone to diffuse out of the cell, was thus correlated with lower leakage.

### Effects of different $p\text{CO}_2$ levels and N sources

The affinity for  $\text{C}_i$  was downregulated at elevated  $p\text{CO}_2$ , as indicated by high  $K_{1/2}$  values under these conditions (Supplementary Figure 1B). Under acclimation DIC, however,  $\text{C}_i$  fluxes (C fixation,  $\text{C}_i$  uptake, and  $\text{CO}_2$  uptake and efflux) were not

**Table 1.** Diurnal cycle of  $\text{C}_i$  fluxes measured by MIMS under acclimation DIC levels (~2100  $\mu\text{mol l}^{-1}$ ) in *Trichodesmium* acclimated to two different  $p\text{CO}_2$  levels (380 vs 1400  $\mu\text{atm}$ ) and N sources ( $\text{N}_2$  vs  $\text{NO}_3^-$ )<sup>a</sup>

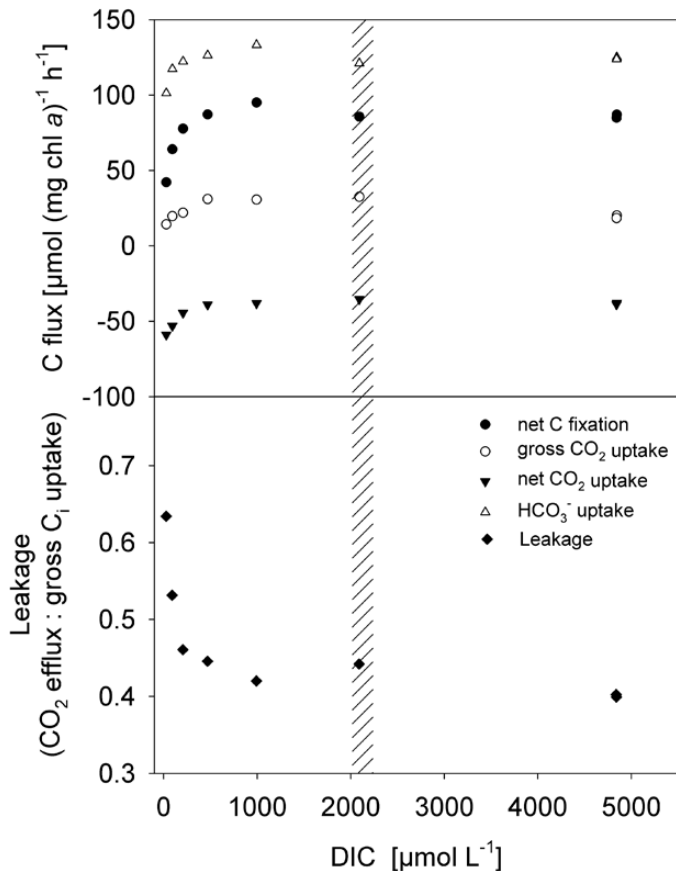
	380 $\mu\text{atm -NO}_3^-$			380 $\mu\text{atm +NO}_3^-$			1400 $\mu\text{atm -NO}_3^-$			1400 $\mu\text{atm +NO}_3^-$		
	Morning	Midday	Evening	Morning	Midday	Evening	Morning	Midday	Evening	Morning	Midday	Evening
Net C fixation	91 ± 15	57 ± 14	87 ± 3	95 ± 20	56 ± 22	70 ± 18	80 ± 13	39 ± 14	61 ± 4	91	61 ± 19	63 ± 16
Gross $\text{C}_i$ uptake	157 ± 18	144 ± 24	167 ± 17	135 ± 23	128 ± 16	143 ± 25	144 ± 7	124 ± 9	142 ± 6	144	127 ± 22	131 ± 17
$\text{HCO}_3^-$ uptake	134 ± 18	117 ± 22	133 ± 12	118 ± 21	108 ± 14	113 ± 14	126 ± 11	95 ± 9	112 ± 2	120	97 ± 17	102 ± 12
Gross $\text{CO}_2$ uptake	23 ± 4	27 ± 4	34 ± 6	17 ± 3	20 ± 2	30 ± 13	18 ± 4	28 ± 5	30 ± 6	24	30 ± 9	29 ± 6
Net $\text{CO}_2$ uptake	-45 ± 10	-60 ± 12	-46 ± 9	-23 ± 2	-52 ± 8	-42 ± 11	-46 ± 5	-56 ± 8	-51 ± 4	-28	-36 ± 8	-39 ± 9
$\text{HCO}_3^-:\text{C}_i$ uptake	0.85 ± 0.03	0.81 ± 0.03	0.80 ± 0.02	0.87 ± 0.01	0.84 ± 0.01	0.80 ± 0.06	0.88 ± 0.03	0.77 ± 0.04	0.79 ± 0.03	0.83	0.76 ± 0.05	0.78 ± 0.01
$\text{CO}_2$ efflux	69 ± 12	87 ± 13	79 ± 13	40 ± 3	72 ± 6	72 ± 8	64 ± 7	85 ± 14	81 ± 3	53	66 ± 4	67 ± 10
Leakage	0.44 ± 0.05	0.61 ± 0.05	0.47 ± 0.03	0.30 ± 0.03	0.57 ± 0.11	0.51 ± 0.04	0.45 ± 0.07	0.69 ± 0.10	0.57 ± 0.01	0.37	0.53 ± 0.07	0.52 ± 0.07

<sup>a</sup> All  $\text{C}_i$  fluxes are given in  $\mu\text{mol C (mg chl a)}^{-1} \text{ h}^{-1}$ . Errors are standard deviations for biological replicates (1 SD;  $n = 3$  except 1400 +  $\text{NO}_3^-$  morning with  $n = 1$ ).

significantly affected by  $p\text{CO}_2$  (ANOVA,  $P > 0.05$ ; Table 1), reflecting the cells' capacity to achieve similar C fixation over a range of  $p\text{CO}_2$  levels by regulating their CCM. Regarding the N source, C fixation rates and  $\text{CO}_2$  uptake under acclimation DIC were equally unaffected (ANOVA,  $P > 0.05$ ; Table 1). Although cells mainly used  $\text{HCO}_3^-$  as a C<sub>i</sub> source in all treatments,  $\text{HCO}_3^-$  uptake at acclimation DIC decreased slightly with increasing  $p\text{CO}_2$  (~10%; ANOVA,  $P < 0.05$ ; Table 1, Supplementary Figure 1C), but was not affected by N source (ANOVA,  $P > 0.05$ ; Table 1). Interestingly,  $\text{CO}_2$  efflux was affected by the N source (ANOVA,  $P < 0.01$ ; Table 1), with ~20% lower efflux in  $\text{NO}_3^-$  users compared to  $\text{N}_2$  fixers, possibly due to differences in internal pH caused by the uptake/accumulation of  $\text{NO}_3^-$  vs  $\text{NH}_4^+$  in the cell. One could also speculate that growing cells on  $\text{NO}_3^-$  reduces the general membrane permeability, since  $\text{NH}_4^+$  transfer between cells is only necessary under  $\text{N}_2$ -fixing conditions, which could also affect the permeability for  $\text{CO}_2$ . Leakage at acclimation DIC estimated by MIMS was, however, not significantly affected by  $p\text{CO}_2$  or N source at any time of the day (ANOVA,  $P > 0.05$ ; Table 1).

### Offsets in leakage estimates

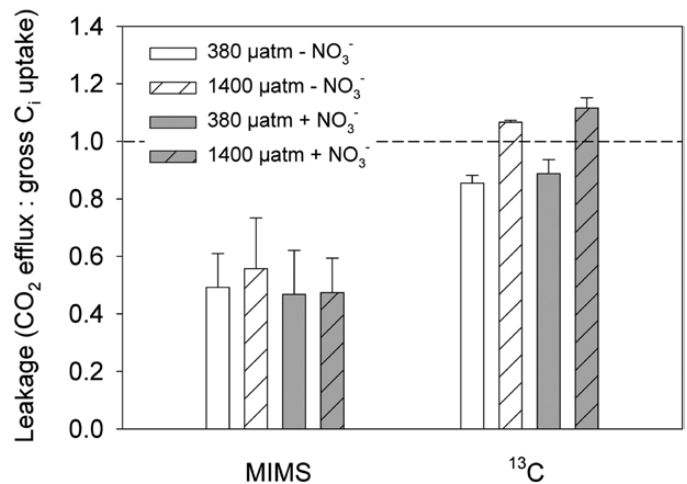
High leakage values obtained in MIMS measurements reflect the strong C<sub>i</sub> accumulation necessary for C fixation



**Fig. 2.** Example showing the dependence of C<sub>i</sub> fluxes measured by MIMS in *Trichodesmium* on the DIC concentration in the assay. Data shown were measured in the evening in a culture grown at 380  $\mu\text{atm}$   $p\text{CO}_2$  without  $\text{NO}_3^-$ . The shaded area denotes the range of acclimation DIC levels.

in cyanobacteria due to the poor  $\text{CO}_2$  affinity of their RubisCO. However, leakage estimates obtained from  $\delta^{13}\text{C}$  values ( $L_{13\text{C}}$ , eqn 3) even exceeded MIMS-based estimates. Overall fractionation during formation of POC ( $\epsilon_p$ ) was not significantly affected by N treatment (ANOVA,  $P > 0.05$ ) but increased with  $p\text{CO}_2$  (ANOVA,  $P < 0.0001$ ), ranging from  $14.4 \pm 1.0\text{‰}$  at 380  $\mu\text{atm}$  to  $19.9 \pm 0.9\text{‰}$  at 1400  $\mu\text{atm}$   $p\text{CO}_2$ . Consequently, leakage estimates based on  $\epsilon_p$  (eqn 3) also increased with  $p\text{CO}_2$ , while estimates from MIMS measurements at acclimation DIC were constant over the range of  $p\text{CO}_2$  levels.  $L_{13\text{C}}$  was calculated to range between 0.82 and 1.14, exceeding MIMS-based measurements by ~30–60% (Fig. 3) and even reaching theoretically impossible values ( $>1$ ). A similar discrepancy between these two approaches, which was equally dependent on  $p\text{CO}_2$  acclimation, has been observed previously (Kranz *et al.*, 2010). In the following paragraph, possible reasons for the deviations between estimates are outlined.

Following the approach by Badger *et al.* (1994), leakage is directly calculated from the measured  $\text{CO}_2$  efflux and gross C<sub>i</sub> uptake. As  $\text{CO}_2$  efflux cannot readily be determined during the light due to the concurrent C<sub>i</sub> uptake, the rise in the  $\text{CO}_2$  signal directly after switching off the light is taken as an estimate of  $\text{CO}_2$  efflux during the light phase, assuming that the accumulated C<sub>i</sub> pool and therewith gross  $\text{CO}_2$  efflux are initially at the pre-darkness level (Badger *et al.*, 1994). If active C<sub>i</sub> uptake as well as C fixation by RubisCO do not cease immediately upon darkening, leakage estimates could be biased and likely to be underestimated. Despite these potential uncertainties, this is a more direct approach than the alternative method, which infers leakage from the isotopic composition of cells. The  $^{13}\text{C}$ -based approach makes use of the effect of leakage on  $\epsilon_p$  (eqn 3; Sharkey and Berry, 1985). Briefly, while the intrinsic fractionation by RubisCO ( $\epsilon_{\text{Rub}}$ ) generally causes organic material to be depleted in  $^{13}\text{C}$  (Fig. 1A), variation in  $\epsilon_p$  can be induced by changes in the C<sub>i</sub> source and/or leakage. Consequently, any errors in



**Fig. 3.** Leakage estimates by MIMS (mean values of measurements conducted at three time points over the day; Badger *et al.*, 1994) and  $^{13}\text{C}$  fractionation (Sharkey and Berry, 1985) determined in *Trichodesmium* grown under two  $p\text{CO}_2$  levels and N sources ( $n \geq 3$ ).

estimates of  $\epsilon_{Rub}$  or  $a_{cyl}$ , but also any unaccounted process affecting  $\epsilon_p$ , would cause  $^{13}\text{C}$ -based leakage estimates to be biased.

Kranz *et al.* (2010) suggested that internal  $\text{C}_i$  cycling within the cell may affect  $\epsilon_p$  in general. The  $\text{CO}_2$  dependence of the offset between MIMS- and  $^{13}\text{C}$ -based leakage estimates was furthermore suggested to reflect a  $\text{CO}_2$  effect on the NDH complex driving this internal  $\text{C}_i$  cycling (Kranz *et al.*, 2010), in line with early observations of the  $\text{C}_i$  dependence of  $\text{CO}_2$  uptake by the NDH complex (Price and Badger, 1989a,c). In Fig. 1, the effects of internal  $\text{C}_i$  cycling on isotopic composition are illustrated. While Fig. 1A assumes an equilibrium situation and does not include any internal  $\text{C}_i$  cycling, Fig. 1B illustrates non-equilibrium situations caused by internal  $\text{C}_i$  cycling. The degree of  $^{13}\text{C}$  enrichment in the cytosol and within the carboxysome, according to this concept, would be dependent on the type of kinetic fractionation in the cytosol. This could include complete or incomplete unidirectional fractionation as well as enzymatic fractionation by the NDH complex. Accounting for these processes requires the introduction of a second compartment. The approach taken here can be considered as an extension of the model of Sharkey and Berry (1985), which considers the cell as one compartment. In order to avoid errors being introduced by large uncertainties, e.g. in permeability of the plasma membrane and carboxysome in *Trichodesmium*, a flux-based model that is independent of these assumptions is employed, rather than a full kinetic model. Our approach is similar to the model of Schulz *et al.* (2007), but disequilibrium situations are also considered.

#### Internal $\text{C}_i$ fluxes and fractionation—model setup

To test our concept (Fig. 1) and quantitatively describe the possible effect of internal cycling on  $\delta^{13}\text{C}$ , intracellular  $\text{C}_i$  fluxes and their effects on isotopic ratios in different cellular  $\text{C}_i$  pools were modelled. For parameterizations,  $\text{HCO}_3^-$  and gross  $\text{CO}_2$  fluxes measured by MIMS as well as measured fractionation values  $\epsilon_p$  were used. The model is based on flux balance equations for the individual isotope species. The flux balance of total C ( $^{12}\text{C} + ^{13}\text{C}$ ) in the cytosol and in the carboxysome, respectively, is given by the following equations:

$$\begin{aligned} F_{\text{cyl},\text{CO}_2} + F_{\text{cyl},\text{HCO}_3^-} + F_{\text{carb},\text{out}} \\ - F_{\text{cyl},\text{out}} - F_{\text{carb},\text{CO}_2} - F_{\text{carb},\text{HCO}_3^-} = 0 \end{aligned} \quad (4)$$

$$F_{\text{carb},\text{HCO}_3^-} + F_{\text{carb},\text{CO}_2} - F_{\text{carb},\text{out}} - F_{\text{fix}} = 0 \quad (5)$$

As about 99% of C is  $^{12}\text{C}$ , i.e.  $F = ^{12}F + ^{13}F \equiv ^{12}F$ , the flux balance equations for  $^{13}\text{C}$  can be derived by multiplying the fluxes ( $F$ ) with the isotopic ratio  $R = ^{13}\text{C}/^{12}\text{C}$ . The isotopic fractionation factor  $\alpha_{db}$  is defined by the isotopic ratio of  $\text{CO}_2$  divided by the isotopic ratio of  $\text{HCO}_3^-$ , i.e.  $\alpha_{db} = R_{\text{CO}_2} / R_{\text{HCO}_3^-}$ . Using the equilibrium fractionation ( $\epsilon_{db}$ ), the fractionation factor between  $\text{CO}_2$  and  $\text{HCO}_3^-$  can be calculated for the external medium as well as for the cytosol according to:

$$\alpha_{db,\text{ext}} = 1 + \epsilon_{db}/1000 \quad (6)$$

$$\alpha_{db,\text{cyl}} = 1 + \epsilon_{\text{cyl}}/1000 \quad (7)$$

While the equilibrium value  $\epsilon_{db}$  is  $-9\text{‰}$  (i.e.  $\text{CO}_2$  is isotopically lighter than  $\text{HCO}_3^-$ ; Mook *et al.*, 1974),  $\epsilon_{\text{cyl}}$  can significantly deviate from this value due to kinetic effects. The uncatalysed conversion of  $\text{HCO}_3^-$  to  $\text{CO}_2$  shows a kinetic fractionation of  $-22\text{‰}$ , whereas the formation of  $\text{HCO}_3^-$  from  $\text{CO}_2$  is associated with a kinetic fractionation of  $+13\text{‰}$  (Marlier and O'Leary, 1984). Hence, the actual value of  $\epsilon_{\text{cyl}}$  is determined by the disequilibrium between  $\text{CO}_2$  and  $\text{HCO}_3^-$  in the cytosol, which depends on all fluxes in and out of the cytosol, and on the internal  $\text{CO}_2$  and  $\text{HCO}_3^-$  concentrations, which cannot be calculated in the framework of a flux-based model. Assuming a unidirectional conversion of  $\text{CO}_2$  to  $\text{HCO}_3^-$  in the cytosol, a value of  $+13\text{‰}$  for  $\epsilon_{\text{cyl}}$  will be adopted. By setting  $\epsilon_{\text{cyl}}$  to  $+30\text{‰}$ , a potential fractionation by the NDH-1<sub>4</sub> complex will be taken into account. The situation where the conversion of  $\text{CO}_2$  to  $\text{HCO}_3^-$  in the cytosol is not completely unidirectional will be considered by setting  $\epsilon_{\text{cyl}}$  to  $+8\text{‰}$ .

The  $R$  associated with  $F_{\text{fix}}$  can be written in terms of the isotopic fractionation against  $^{13}\text{C}$  by RubisCO described by the factor  $\alpha_{Rub} = R_{\text{carb}} / R_{\text{POC}}$ , where  $R_{\text{carb}}$  is the isotopic ratio of  $\text{CO}_2$  in the carboxysome and  $R_{\text{POC}}$  is the isotopic ratio of POC. The value of  $\alpha_{Rub}$  is calculated from the intrinsic RubisCO fractionation  $\epsilon_{Rub}$  (assuming an intermediate value of  $+25\text{‰}$ ; Roeske and O'Leary, 1984; Guy *et al.*, 1993):

$$\alpha_{Rub} = 1 + \epsilon_{Rub}/1000 \quad (8)$$

Given the isotopic ratios ( $R$ ) of  $\text{CO}_2$  and the isotopic fractionation factors between  $\text{HCO}_3^-$  and  $\text{CO}_2$  expressed as  $\alpha_{bd} = 1/\alpha_{db}$ , the flux balance equations for  $^{13}\text{C}$  can be derived from eqns 4 and 5 for the cytosol and the carboxysome, respectively:

$$\begin{aligned} R_{\text{ext}}F_{\text{cyl},\text{CO}_2} + \alpha_{bd,\text{ext}}R_{\text{ext}}F_{\text{cyl},\text{HCO}_3^-} + R_{\text{carb}}F_{\text{carb},\text{out}} \\ - R_{\text{cyl}}F_{\text{cyl},\text{out}} - R_{\text{cyl}}F_{\text{carb},\text{CO}_2} - \alpha_{bd,\text{cyl}}R_{\text{cyl}}F_{\text{carb},\text{HCO}_3^-} = 0 \end{aligned} \quad (9)$$

$$\begin{aligned} \alpha_{bd,\text{cyl}}R_{\text{cyl}}F_{\text{carb},\text{HCO}_3^-} + R_{\text{cyl}}F_{\text{carb},\text{CO}_2} \\ - R_{\text{carb}}F_{\text{carb},\text{out}} - R_{\text{carb}}F_{\text{fix}}/\alpha_{Rub} = 0 \end{aligned} \quad (10)$$

$R_{\text{cyl}}$  is the isotopic ratio of  $\text{CO}_2$  in the cytosol. The overall isotopic fractionation by the cell is defined with respect to the isotopic composition of  $\text{CO}_2$  in the external medium ( $R_{\text{ext}}$ ):

$$\epsilon_p = \left( \frac{R_{\text{ext}}}{R_{\text{POC}}} - 1 \right) \times 1000 = \left( \alpha_{Rub} \frac{R_{\text{ext}}}{R_{\text{carb}}} - 1 \right) \times 1000 \quad (11)$$

The ratio  $R_{\text{ext}}/R_{\text{carb}}$  reflects the impact of the inner compartment on the isotopic fractionation and can be calculated from flux balance eqns 9 and 10. Eqn 10 can be solved for  $R_{\text{cyl}}$ , which in turn is substituted into eqn 9, yielding the ratio:



$$\begin{aligned}
\frac{R_{ext}}{R_{carb}} &= \frac{(F_{fix}/\alpha_{Rub} + F_{carb,out})(F_{carb,CO_2} + \alpha_{bd,cyt}F_{carb,HCO_3^-} + F_{cyt,out})}{(F_{cyt,CO_2} + \alpha_{bd,ext}F_{cyt,HCO_3^-})(F_{carb,CO_2} + \alpha_{bd,cyt}F_{carb,HCO_3^-})} - \frac{F_{carb,out}}{(F_{cyt,CO_2} + \alpha_{bd,ext}F_{cyt,HCO_3^-})} \\
&= \frac{F_{cyt,out}}{\alpha_{Rub}(F_{cyt,CO_2} + \alpha_{bd,ext}F_{cyt,HCO_3^-})} \times \left( \frac{F_{fix}}{F_{cyt,out}} + \frac{F_{fix} + \alpha_{Rub}F_{carb,out}}{F_{carb,CO_2} + \alpha_{bd,cyt}F_{carb,HCO_3^-}} \right)
\end{aligned} \tag{12}$$

This solution is valid for arbitrary combinations of fluxes as long as the constraints imposed by flux balance equations 4 and 5 are obeyed:

$$\begin{aligned}
F_{fix} &= F_{carb,CO_2} + F_{carb,HCO_3^-} - F_{carb,out} \\
&= F_{cyt,CO_2} + F_{cyt,HCO_3^-} - F_{cyt,out}
\end{aligned} \tag{13}$$

Given the fractional contribution of HCO<sub>3</sub><sup>-</sup> to total C<sub>i</sub> uptake into the cytosol ( $a_{cyt}$ ) and the carboxysome ( $a_{carb}$ ), as well as the leakage out of the cytosol ( $L_{cyt}$ ) and the carboxysome ( $L_{carb}$ ), eqns 6 to 8 and 11 to 13 can be used to derive the overall isotopic fractionation:

$$\begin{aligned}
\varepsilon_p &= \frac{a_{cyt}\varepsilon_{db}}{1 - a_{cyt}\varepsilon_{db}/10^3} + L_{cyt} \frac{(a_{carb}\varepsilon_{cyt} + L_{carb}\varepsilon_{Rub})}{(1 - a_{cyt}\varepsilon_{db}/10^3)(1 - a_{carb}\varepsilon_{cyt}/10^3)} \\
&\approx a_{cyt}\varepsilon_{db} + L_{cyt}(a_{carb}\varepsilon_{cyt} + L_{carb}\varepsilon_{Rub}).
\end{aligned} \tag{14}$$

Solving the approximated solution for  $L_{cyt}$  yields the following:

$$L_{cyt} = \frac{\varepsilon_p - a_{cyt}\varepsilon_{db}}{a_{carb}\varepsilon_{cyt} + L_{carb}\varepsilon_{Rub}} \tag{15}$$

The approximate solution can be considered as a generalization of the original function given by Sharkey and Berry (1985), accounting for two compartments. The authors assumed that the cell takes up HCO<sub>3</sub><sup>-</sup> into a single compartment and subsequently converts it to CO<sub>2</sub>; hence there is no HCO<sub>3</sub><sup>-</sup> inside the cell. The compatibility of our model with the original function can be confirmed by comparing  $\varepsilon_p$  for  $L_{carb} = 1$  (i.e. no second compartment) and  $a_{carb} = 0$  (i.e. only CO<sub>2</sub> uptake into the carboxysome).

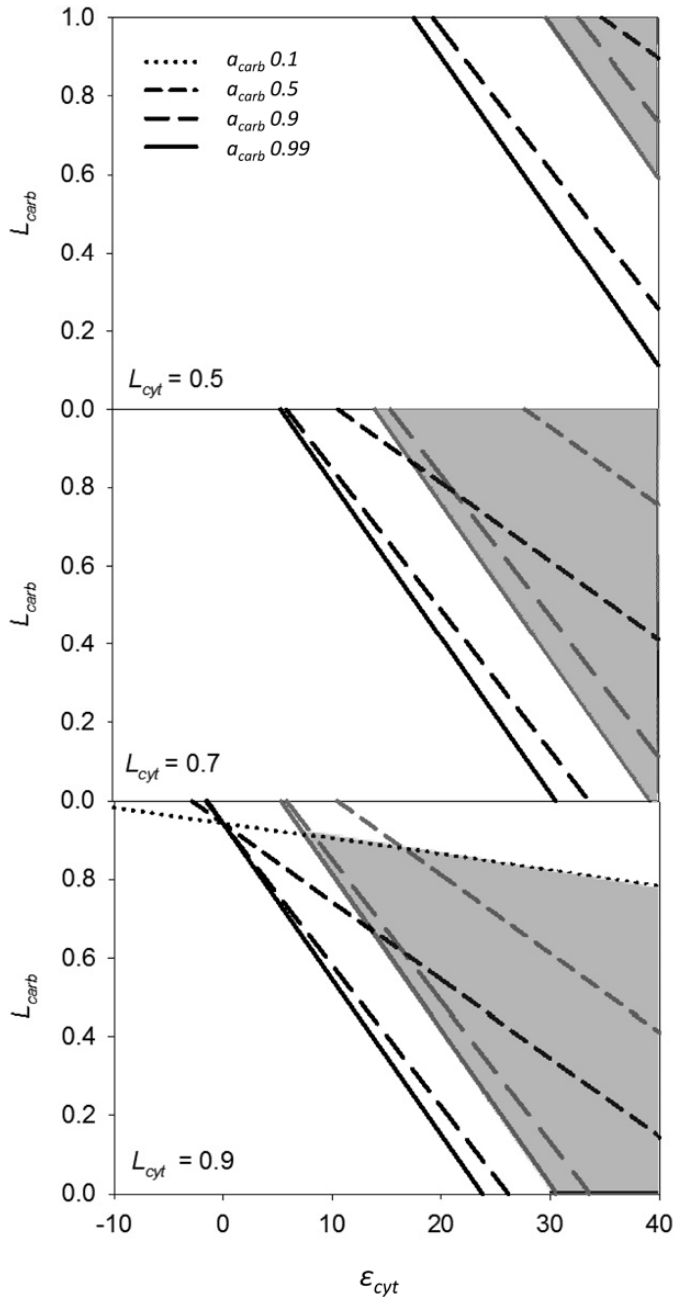
As pointed out by Schulz *et al.* (2007), diffusive CO<sub>2</sub> fluxes generally need to be added to cellular fluxes measured by MIMS (Badger *et al.*, 1994) when relating them to <sup>13</sup>C fractionation. For membrane permeability exceeding 10<sup>-4</sup> cm s<sup>-1</sup>, as proposed for a diatom (~10<sup>-2</sup> cm s<sup>-1</sup>; Hopkinson *et al.*, 2011), diffusive CO<sub>2</sub> fluxes are high and internal CO<sub>2</sub> concentrations approach those of the cell's exterior (Supplementary Figure S2). In this case, gross CO<sub>2</sub> efflux estimated by MIMS would be underestimated, which could explain part of the discrepancy between MIMS-based leakage and estimates based

on eqn 4 (Sharkey and Berry, 1985). While there is, to our knowledge, no recent data on the membrane permeability of cyanobacteria available, older studies on cyanobacteria state significantly lower values, approaching 10<sup>-5</sup> cm s<sup>-1</sup> (Badger *et al.*, 1985; Marcus *et al.*, 1986), which are in line with diffusive CO<sub>2</sub> fluxes being low enough to allow for considerable CO<sub>2</sub> accumulation in the cell (Supplementary Figure S2). Using this permeability, the effect of diffusive CO<sub>2</sub> influx on leakage obtained by our model was estimated, yielding maximum changes in the order of a few percent, which were thus neglected. In view of the uncertainties in this parameter, measurements of membrane permeability of cyanobacteria are needed to improve future estimates of internal C fluxes.

#### Internal C<sub>i</sub> fluxes and fractionation—model application

To test the sensitivity of our model, the potential effect of changes in  $a_{cyt}$  on  $\varepsilon_p$  was quantified, using the maximum variability observed in our study (0.84 vs 0.76) while leaving all other parameters constant. This variability can explain a change in  $\varepsilon_p$  by not more than 0.7‰. Thus,  $a_{cyt}$  can be excluded as a main driver behind the variability in  $\varepsilon_p$  (or leakage estimates), even if variability in  $a_{cyt}$  is severely underestimated. Applying the model to our measured fluxes and  $\varepsilon_p$  values, a range of different possible scenarios for intracellular fluxes and fractionation in the cytosol is obtained (Fig. 4).

According to these interrelations, while at  $L_{cyt}$  according to our MIMS measurements (0.5), only a very high fractionation in the cytosol ( $\varepsilon_{cyt}$ ) can explain our results, at  $L_{cyt} \geq 0.7$ , there is a large range of possible combinations of parameters (see shaded areas in Fig. 4). As we aim to find parameters that can explain  $\varepsilon_p$  in both of our pCO<sub>2</sub> treatments, the high  $\varepsilon_p$  measured in cells grown at 1400 μatm constrains the range of possible values, while  $\varepsilon_p$  of cells grown at 380 μatm could be explained by a larger range of values for  $a_{carb}$  and  $L_{carb}$  (Fig. 4). High values for  $a_{carb}$  and  $L_{carb}$  (both approaching 1) allow for a larger range of possible values of  $\varepsilon_{cyt}$  to explain our measured  $\varepsilon_p$  (Fig. 4). Due to the high contribution of HCO<sub>3</sub><sup>-</sup> to C<sub>i</sub> uptake and the additional conversion of CO<sub>2</sub> to HCO<sub>3</sub><sup>-</sup> by the NDH complex,  $a_{carb}$  is likely to be close to 1, most probably exceeding  $a_{cyt}$  measured in our experiment (0.82). Moreover, high diffusive CO<sub>2</sub> influx into the carboxysome seems unlikely in view of the supposed function of the carboxysome as a diffusion barrier to CO<sub>2</sub> (e.g. Reinhold *et al.*, 1989). While comparison experiments with CA knockout mutants with intact and broken carboxysomes confirmed that the carboxysome shell impedes diffusion of CO<sub>2</sub> (Dou *et al.*, 2008), the pores in the hexamer protein subunits of the shell are supposed to



**Fig. 4.** Interrelationship between  $L_{carb}$ ,  $\epsilon_{cyt}$ , and  $a_{carb}$  in the model, depicted for different values of  $L_{cyt}$  and  $\epsilon_p$ . The shaded areas mark the range of possible values for  $L_{carb}$  and  $\epsilon_{cyt}$  that could reconcile our measurements of isotopic composition with measured external  $C_i$  fluxes. Black and grey lines are based on  $\epsilon_p$  measured in cells acclimated to 380 and 1400  $\mu\text{atm } p\text{CO}_2$ , respectively.

be permeable to small, negatively charged molecules such as  $\text{HCO}_3^-$  (Tsai et al., 2007; Klein et al., 2009; Espie and Kimber, 2011). Despite the low  $\text{CO}_2$  permeability, high rates of  $\text{CO}_2$  efflux, and thus high  $L_{carb}$ , are likely due to the very high accumulation factor (two to three orders of magnitude; this study and Kaplan et al., 1980). A value for  $L_{carb}$  of 0.9 is therefore used in the model scenarios described in the following (Table 2). Using eqn 13, the following expression for the ratio of internal to external  $C_i$  fluxes can be derived:

**Table 2.** Different scenarios of external and internal  $C_i$  fluxes that can reconcile measurements of  $C_i$  fluxes by MIMS and  $\epsilon_p$  values obtained in this study (scenarios 1, 2 and 4 to 6) and by Kranz et al. (2010, scenario 3)<sup>a</sup>

Scenario	$p\text{CO}_2$	$\epsilon_p$	$a_{cyt}$	$L_{MIMS}$	$L_{13C}$	$L_{cyt}$				$\epsilon_{cyt}$
						$L_{carb}$	$a_{carb}$	Modelled		
1	1400	20	0.8	0.5	1.1	0.8	0.9	1	13	
2	380	14	0.8	0.5	0.8	0.6	0.9	1	13	
3	180	7	0.8	0.4	0.6	0.4	0.9	1	13	
4	1400	20	0.8	0.5	1.1	0.9	0.9	1	8	
5	1400	20	0.8	0.5	1.1	0.5	0.9	1	30	
6	380	14	0.8	0.5	1.1	0.6	0.9	0.7	20	

<sup>a</sup> $\epsilon_p$ ,  $a_{cyt}$ , and  $L_{MIMS}$  were measured;  $L_{13C}$  was calculated from  $\epsilon_p$  according to Sharkey and Berry (1985); remaining values are model input parameters and model results ( $L_{cyt}$ ,  $L_{carb}$ ,  $a_{carb}$ , and  $\epsilon_{cyt}$ ).

$$\frac{F_{carb,CO_2} + F_{carb,HCO_3^-}}{F_{cyt,CO_2} + F_{cyt,HCO_3^-}} = \frac{1 - L_{cyt}}{1 - L_{carb}} \quad (16)$$

For the chosen value for  $L_{carb}$  of 0.9 and the measured  $L_{cyt}$  of 0.5, eqn 16 yields a ratio of internal vs external  $C_i$  cycling of 5.

Compared to estimates based on Sharkey and Berry (1985), our model significantly improved the compatibility of leakage estimates with those obtained by MIMS measurements (Table 2). The maximum fractionation that could be achieved in an uncatalysed reaction from  $\text{CO}_2$  to  $\text{HCO}_3^-$  is +13‰ (O’Leary et al., 1992). With this kinetic fractionation,  $\epsilon_p$  values measured for the two  $p\text{CO}_2$  levels can be explained by leakage values from the cytosol ( $L_{cyt}$ ) of 0.8 and 0.6, respectively (scenarios 1 and 2, Table 2), which are significantly lower than the estimates based on the function by Sharkey and Berry ( $L_{13C} = 1.1$  and 0.8, respectively). The remaining difference to leakage estimates obtained by MIMS ( $L_{MIMS} = 0.5$ ) could be explained by an underestimation of leakage by the MIMS approach, as discussed above. Assuming that the conversion between  $\text{CO}_2$  and  $\text{HCO}_3^-$  in the cytosol was not completely unidirectional,  $\epsilon_{cyt}$  could range between +13‰ and -9‰ (equilibrium fractionation; O’Leary et al., 1992). To simulate this intermediate scenario, an  $\epsilon_{cyt}$  of +8‰ is assumed (scenario 4, Table 2), yielding an  $L_{cyt}$  of 0.9 for the high  $\text{CO}_2$  treatment.

Kinetic fractionation could be achieved by the NDH complex or by creation of a strong disequilibrium in the cytosol, minimizing the back-reaction from  $\text{HCO}_3^-$  to  $\text{CO}_2$ . Mutants of *Synechococcus* expressing human CA in the cytosol were unable to accumulate  $C_i$  (Price and Badger, 1989b), suggesting that  $\text{HCO}_3^-$  is accumulated in the cytosol, and that a chemical disequilibrium in the cytosol favours the reaction from  $\text{HCO}_3^-$  to  $\text{CO}_2$  rather than the opposite direction. This strongly argues for a fractionating enzyme instead of a purely chemical disequilibrium driving unidirectional  $\text{CO}_2$  to  $\text{HCO}_3^-$  conversion in the cytosol. Assuming that NDH not only drives the unidirectional conversion of  $\text{CO}_2$  to  $\text{HCO}_3^-$  but also discriminates against  $^{13}\text{C}$  during the reaction, leakage estimates by our model can be further reconciled with MIMS-based



estimates. In a scenario assuming an upper estimate for  $\epsilon_{\text{cyt}}$  of +30‰ (scenario 5, Table 2), which is within the range of fractionation measured in other enzymes such as RubisCO, our MIMS-measured data can be reproduced even for the high  $p\text{CO}_2$  treatment ( $L_{\text{MIMS}} = L_{\text{cyt}} = 0.5$ ; Table 2). Note that in combination with one of the other factors discussed above, such as an underestimation of  $L_{\text{carb}}$  or of MIMS-based leakage estimates, enzymatic fractionation less than +30‰ could also explain our measurements (cf. e.g. scenarios with  $\epsilon_{\text{cyt}}$  of 13‰; Table 2). Although CA and NDH have been proposed to have similar reaction mechanisms (Price *et al.*, 2002), our model results suggest that the fractionation by the NDH complex is different from that of CA (1‰ for conversion of  $\text{CO}_2$  to  $\text{HCO}_3^-$ ; Paneth and O'Leary, 1985). This might be due to the fact that the subunit carrying out the hydration reaction in the NDH-1<sub>4</sub> complex (chpX) is embedded in a larger functional unit including the transmembrane proton channel and is associated with the electron transport chain. To confirm the differences in fractionation between these enzyme complexes, however, further work using experimental approaches would be necessary, e.g. by comparing cyanobacterial mutant strains.

Since our MIMS measurements showed that leakage was unaffected by  $p\text{CO}_2$  and the slight changes in  $a_{\text{cyt}}$  could not explain the observed variation in  $\epsilon_p$ ,  $a_{\text{carb}}$  and/or  $\epsilon_{\text{cyt}}$  would need to vary with  $p\text{CO}_2$  to explain the observed  $\text{CO}_2$ -dependence of  $\epsilon_p$ . An increase in the activity of the NDH complex could yield an increase in  $a_{\text{carb}}$  as well as  $\epsilon_{\text{cyt}}$  and therewith  $\epsilon_p$ . With an increase in  $a_{\text{carb}}$  from 0.7 to 1 and an increase in  $\epsilon_{\text{cyt}}$  from 20 to 30‰, the measured increase in  $\epsilon_p$  between 380 and 1400  $\mu\text{atm}$  can be explained, almost reproducing MIMS-measured leakage in both scenarios (scenarios 5 and 6, Table 2). A higher activity of NDH at high  $p\text{CO}_2$  may seem unexpected in view of its supposed role as part of the CCM. However, in contrast to other components of the CCM such as  $\text{HCO}_3^-$  transporters, the reaction catalysed by the NDH complex contributes to ATP regeneration rather than consuming energy, and thus a downregulation of NDH at high  $p\text{CO}_2$  would not provide any energetic benefit to the cell. A positive correlation with  $p\text{CO}_2$  might be coupled to the small  $\text{CO}_2$  effect on  $a_{\text{cyt}}$  (Table 1; Supplementary Figure 1C), increasing the activity of the NDH complex at high  $p\text{CO}_2$  levels in the acclimations in response to a higher availability of its substrate  $\text{CO}_2$ . Note that the change in  $a_{\text{cyt}}$  may have been underestimated in our study due to the constant pH in MIMS measurements. Due to its function as a proton pump in the thylakoid membrane, activity of the NDH complex can increase the ratio of ATP to NADPH available in the cell. The production of ATP by high NDH activity at high  $p\text{CO}_2$  could, in turn, contribute to the increased ATP requirement to fuel  $\text{N}_2$  fixation in *Trichodesmium* under ocean acidification (e.g. Kranz *et al.*, 2010; Eichner *et al.*, 2014).

Kranz *et al.* (2010) compared leakage estimates based on MIMS and  $^{13}\text{C}$  for *Trichodesmium* grown under different  $p\text{CO}_2$  levels as well as light intensities. Applying our model to this data set, MIMS-based leakage estimates could be reproduced with unidirectional fractionation for a low  $p\text{CO}_2$  treatment with light intensities similar to our experiment

(200  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ; scenario 3, Table 2). In high  $p\text{CO}_2$  and low light treatments, the difference between  $L_{\text{MIMS}}$  and  $L_{\text{cyt}}$  was larger and could consequently only be reconciled with  $\epsilon_{\text{cyt}}$  values larger than +30‰. Short-term exposure to high light intensities (300  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) in our experiment affected  $\text{CO}_2$  efflux in cells acclimated to high  $p\text{CO}_2$  (ANOVA,  $P < 0.05$ ; data not shown). Such light sensitivity generally suggests  $\text{CO}_2$  efflux to be closely associated to photosynthetic electron transport. As this light effect was only observed under high  $p\text{CO}_2$ , i.e. conditions associated with higher NDH activity according to our model results, the interrelation of  $\text{CO}_2$  efflux and the NDH complex is further corroborated. The observed light effects on  $\text{CO}_2$  efflux (this study) and  $\epsilon_p$  (Kranz *et al.*, 2010) impose interesting questions with regard to a potential regulation of the NDH complex by the electron transport chain (redox state and/or proton gradient), which should be investigated in future studies. A better understanding of the regulation of the NDH complex is essential to improve confidence in explaining the effects of  $p\text{CO}_2$  as well as light on internal C<sub>i</sub> fluxes and potential feedbacks on cellular energy budgets of this key  $\text{N}_2$  fixer.

### Conclusion and outlook

This study demonstrates that internal C<sub>i</sub> fluxes via the NDH-1<sub>4</sub> complex need to be considered not only in terms of cellular C<sub>i</sub> acquisition, but also with regard to  $^{13}\text{C}$  fractionation and cellular energy status. The comparison of direct measurements of C<sub>i</sub> fluxes with estimates based on isotopic composition revealed that  $^{13}\text{C}$  fractionation in *Trichodesmium* cannot be adequately described by only considering external C<sub>i</sub> fluxes.

Compatibility with direct leakage measurements was improved by applying a model accounting for internal C<sub>i</sub> fluxes around the carboxysome, e.g. via the NDH complex, providing a generalization of the model of Sharkey and Berry (1985) applicable for two compartments. In these model calculations, a large fraction of  $\text{HCO}_3^-$  uptake into the carboxysome (0.7–1, Table 2) and high leakage from the carboxysome (>0.9, Table 2) are assumed, in accordance with the current understanding of carboxysome functioning. A large range of fractionation values for the cytoplasm, representing uncatalysed, unidirectional fractionation as well as enzymatic fractionation by the NDH complex, could significantly improve compatibility of leakage estimates (Table 2), even though the exact interplay of these processes still has to be resolved. While the lack of recent literature data on membrane permeability of cyanobacteria clearly demands future measurements, our model calculations are insensitive to this parameter within a certain range of permeability estimates ( $<10^{-4} \text{ m}^{-2} \text{ s}^{-1}$ ). Similarly, the model is independent of other potentially uncertain assumptions such as permeability of the carboxysome and pH values for the different compartments. Once future studies improve confidence in these parameters, a kinetic model can be used to predict internal concentrations and individual C<sub>i</sub> fluxes. The agreement of model results with measured values could further be improved by accounting for possible  $^{13}\text{C}$  enrichment of  $\text{CO}_2$

leaking out of the cell as well as a disequilibrium situation in the surroundings of the cell (altering the isotopic signature of  $\text{HCO}_3^-$  taken up).

The model applied here could also be used to improve estimates of leakage based on  $^{13}\text{C}$  signatures for other species in which several compartments and/or internal  $\text{C}_i$  fluxes play an important role. For phytoplankton groups that are relevant in terms of paleo-proxies, this could have important implications for the interpretation of C isotope signals.

## Supplementary material

**Supplementary Figure S1.** DIC-saturated rates of C fixation ( $V_{\text{max}}$ ), half saturation DIC concentrations ( $K_{1/2}$ ) and  $\text{HCO}_3^- : \text{C}_i$  uptake ratios measured at three time points during the day in *Trichodesmium* grown under two  $p\text{CO}_2$  levels and N sources ( $\text{N}_2$  and  $\text{NO}_3^-$ ).

**Supplementary Figure S2.** The dependence of intracellular  $\text{CO}_2$  concentrations on membrane permeability.

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