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Light- and CO₂-dependent carbon isotope fractionation in the β -cyanobacterium *Synechococcus* sp. PCC 7002

Authors: Sarah J. Hurley¹, Boswell A. Wing², Henry C. Holm¹, Ana Gonzalez-Nayeck³, Benjamin L. Acosta¹, Claire E. Jasper^{2,5}, Chloe Huntzinger², Nicholas C. Hill⁴, Margaret Habib⁴, Jeffrey C. Cameron⁴

Affiliations:

¹ Division of Biology and Paleo Environment, Lamont-Doherty Earth Observatory of Columbia University, Palisades, NY 10964

² Department of Earth Science, University of Colorado Boulder, Boulder, CO 80302, USA.

³ Department of Natural Sciences, Baruch College of the City University of New York, New York, NY 10010

⁴ Department of Biochemistry, Renewable and Sustainable Energy Institute, University of Colorado Boulder, Boulder, CO 80302, USA.

⁵ Current affiliation: Ocean Sciences Department, University of California, Santa Cruz, 1156 High Street, Santa Cruz, CA 95064, USA.

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*Corresponding author: Sarah Hurley

Email: shurley@ldeo.columbia.edu

ORCID: <https://orcid.org/0000-0002-4663-4063>

1. Abstract

Carbon isotope fractionation during photosynthetic carbon fixation by phytoplankton ($\epsilon_p \approx \delta^{13}C_{CO_2(aq)} - \delta^{13}C_{biomass}$) underpins proxies for reconstructing past atmospheric CO₂ concentrations. The interpretation of ϵ_p is commonly based on steady-state flux-balance models that link isotopic fractionation to the balance between cellular carbon demand and supply, with Rubisco carboxylation providing the primary fractionating step. Accumulating evidence shows that ϵ_p is also sensitive to other environmental factors, including irradiance, complicating mechanistic interpretation and proxy applications. Previous measurements of cyanobacterial ϵ_p have also been difficult to reconcile with the steady-state flux-balance framework because they span a narrow range and reach maximum values below the Rubisco kinetic isotope effect expected under conditions of high diffusive CO₂ supply. Here, we combined previously published high-irradiance measurements with new low-irradiance experiments to test how irradiance, CO₂ concentration, and carbon-concentrating mechanism (CCM) function affect ϵ_p in the β -cyanobacterium *Synechococcus* sp. PCC 7002. We compared a wild-type strain with a Δccm mutant lacking a functional CCM across multiple CO₂ conditions. In the wild type, ϵ_p varied by approximately 14‰ across treatments and responded strongly to both CO₂ and irradiance. Under high irradiance, ϵ_p increased with increasing CO₂, consistent with conventional steady-state flux-balance predictions, whereas under low irradiance, ϵ_p decreased with increasing CO₂, reversing the expected response. The largest ϵ_p changes occurred when the availability of the factor constraining growth (e.g., carbon availability or light) changed and followed the

direction predicted by the flux-balance framework. However, ϵ_p also increased with irradiance at elevated CO_2 despite substantially faster growth. In contrast, the Δccm mutant exhibited a narrow ϵ_p range of approximately 1–2‰ across the three measured conditions. Maximum ϵ_p values were similar in the wild-type and Δccm strains at approximately 22‰ and were consistent with kinetic isotope effects measured for β -cyanobacterial Rubiscos. Our results indicate that, in *Synechococcus sp. PCC 7002*, the response of ϵ_p to CO_2 depends on irradiance and that loss of CCM function substantially reduces the sensitivity of ϵ_p to both variables. More broadly, the flux-balance framework captured the direction of the largest ϵ_p responses but did not explain the full response across irradiance and CO_2 conditions. Since irradiance changed the direction of the ϵ_p response to CO_2 , changes in the light environment of organic-carbon production could affect the interpretation of ϵ_p -based $p\text{CO}_2$ proxy records.

2. Introduction

Carbon isotope fractionation during photosynthetic carbon fixation ($\epsilon_p \approx \delta^{13}\text{C}_{\text{CO}_2(\text{aq})} - \delta^{13}\text{C}_{\text{biomass}}$) forms the basis of proxies used to reconstruct past atmospheric CO_2 concentrations (Freeman and Hayes, 1992; Rae et al., 2021). During carbon fixation, the stable isotopes of carbon (^{12}C and ^{13}C) are sorted, resulting in biomass that is enriched in ^{12}C relative to inorganic carbon substrates. The magnitude of this fractionation is commonly expressed as ϵ_p , calculated from the isotopic compositions of dissolved CO_2 and phytoplankton biomass. Because ϵ_p frequently increases with increasing CO_2 availability (Bidigare et al., 1997; Popp et al., 1998), relationships between ϵ_p and environmental CO_2 have been employed to reconstruct past atmospheric $p\text{CO}_2$ (Jasper et al., 1994; Freeman and Pagani, 2005; Zhang et al., 2013; Witkowski et al., 2018). These proxies use different molecular fossils derived from phytoplankton, including alkenones produced by haptophyte algae and phytane derived from the phytol side chain of chlorophyll. Because chlorophyll is widespread, phytane can reflect a broader range of algae and cyanobacteria. Bulk organic matter preserves complementary records of carbon isotope fractionation by phytoplankton throughout much of Earth's history (Krissansen-Totton et al., 2015; Hurley et al., 2021).

The classical framework underlying the interpretation of ϵ_p values is a steady-state flux-balance model (Farquhar et al., 1982; Rau et al., 1992; Hayes, 1993; Francois et al., 1993; Goericke et al., 1994; Laws et al., 1995). In this model, cellular carbon isotope fractionation reflects the balance between carbon transport across the cell boundary and carbon fixation by Rubisco. This model assumes that a fraction of inorganic carbon entering the cell is subsequently lost rather than fixed, commonly termed 'leakage'. Variation in leakage results in an expressed isotopic fractionation that varies between two end members: (1) the isotope effect associated with transport (ϵ_t); and (2) the kinetic isotope effect (KIE) associated with Rubisco carboxylation (ϵ_f). Consequently, ϵ_p is expected to vary with the balance between cellular carbon demand and supply, commonly approximated by $\mu/[\text{CO}_2(\text{aq})]$, where μ is the specific growth rate, a proxy for carbon demand, and $[\text{CO}_2(\text{aq})]$ is the dissolved CO_2 concentration in the external medium (Francois et al., 1993; Goericke et al., 1994; Laws et al., 1995; Bidigare et al., 1997; Popp et al., 1998). When CO_2 supply is high relative to cellular demand, ϵ_p approaches the isotope effect of Rubisco carboxylation (ϵ_f). As cellular demand increases relative to CO_2 supply, ϵ_p shifts toward the isotope effect associated with carbon transport (ϵ_t).

Although the flux-balance model is consistent with the observed relationships between ϵ_p and CO_2 across several phytoplankton taxa, accumulating evidence indicates that ϵ_p is also sensitive to irradiance, which

the model accounts for only indirectly through its effect on growth rate. Culturing studies show that ϵ_p varies with irradiance, including by approximately 5–7‰ in the haptophyte *Gephyrocapsa huxleyi* (Thompson and Calvert, 1995; Rost et al., 2002; Trimbom et al., 2007; Pearson et al., 2025). Irradiance is also associated with substantial variation in ϵ_p in the natural environment. In the subtropical North Atlantic, ϵ_p increased by 5.6‰ between the upper and lower euphotic zones (Henderson et al., 2024). This difference was associated with changes in irradiance and phytoplankton community composition but could not be explained by existing flux-balance models. Although the effects of light and community composition could not be separated in the marine water column, these results show that ϵ_p can vary substantially under the same atmospheric CO_2 conditions.

Early applications of the flux-balance framework identified cyanobacteria as an exception to patterns observed in other phytoplankton (Popp et al., 1998). In the marine α -cyanobacterium *Synechococcus* sp. CCMP838, ϵ_p responded only weakly to changes in CO_2 supply relative to cellular demand. This weak response was attributed to the strain's small cell size and consequently high surface-area-to-volume ratio, which could facilitate efficient diffusive CO_2 supply (Popp et al., 1998; Figure 1A). Published ϵ_p values from β -cyanobacteria span a much wider range than those reported for α -cyanobacteria and extend to values near the kinetic isotope effect of Form IB Rubisco (Figure 1A). In a direct test of the CO_2 response, previous experiments with the β -cyanobacterium *Synechococcus* sp. PCC 7002 under high irradiance showed that ϵ_p increased with CO_2 and approached estimates of the kinetic isotope effect of Form IB Rubisco (Hurley et al., 2021). Therefore β -cyanobacteria do not invariably exhibit the weak CO_2 response observed in α -cyanobacteria. However, because those experiments were conducted at a single, saturating irradiance, it remained unclear whether the response of β -cyanobacterial ϵ_p to CO_2 additionally changes with irradiance.

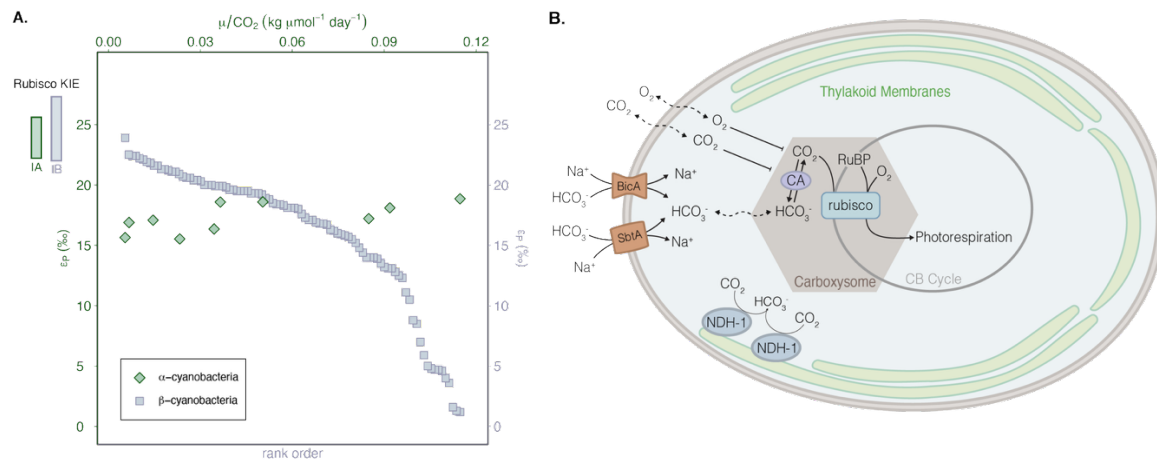


Figure 1. A. Carbon isotope fractionation (ϵ_p) in β -cyanobacteria (blue squares) and α -cyanobacteria (green diamonds). The green rectangle indicates the 95% confidence interval of 22.2–25.6‰ around the 24.0‰ kinetic isotope effect measured for Form IA Rubisco from *Prochlorococcus marinus* MIT9313 (Scott et al., 2007). The blue rectangle indicates the 22.0–27.3‰ range of kinetic isotope effects measured for Form IB Rubisco from β -cyanobacteria (Guy et al., 1993; Wijker et al., 2026; Aguiló-Nicolau et al., 2026). The similarity between these Rubisco isotope effects suggests that differences in Rubisco fractionation alone do not explain the contrasting ϵ_p distributions in α - vs. β -cyanobacteria. α -cyanobacterial ϵ_p values are from Popp et al. (1998). β -cyanobacterial ϵ_p

values were compiled from Calder and Parker (1973), Pardue et al. (1976), Mizutani and Wada (1982), Falkowski (1991), Sakata et al. (1997), and Eichner et al. (2015). **B.** Schematic of a cyanobacterial CO₂-concentrating mechanism, including active HCO₃⁻ transporters, NDH-1 CO₂-uptake complexes, and encapsulation of Rubisco within carboxysomes. Differences in CCM function provide a potential physiological explanation for variation in ϵ_p .

The energetic requirements and light-dependent regulation of cyanobacterial CO₂-concentrating mechanisms (CCMs) provide a potential mechanism through which irradiance could alter the response of ϵ_p to CO₂. Cyanobacterial CCMs combine active bicarbonate transport, NAD(P)H dehydrogenase mediated CO₂ uptake, intracellular interconversion of inorganic carbon, and compartmentalization of Rubisco within carboxysomes (Figure 1B). CCMs therefore regulate the form and flux of inorganic carbon supplied to Rubisco, as well as carbon recycling and loss from the cell. Although traditional flux balance models represent net leakage, they do not explicitly represent active carbon uptake, intracellular inorganic carbon transformations, or isotope effects associated with energy dependent inorganic carbon transport (Keller and Morel, 1999; Wilkes and Pearson, 2019; Wang et al., 2023). Previous high irradiance experiments demonstrated that loss of CCM function alters ϵ_p in *Synechococcus* 7002 (Hurley et al., 2021), but whether CCM function contributes to the response of ϵ_p across irradiance conditions has not been tested.

Synechococcus sp. PCC 7002 (hereafter *Synechococcus* 7002) has a well-characterized CCM and can be compared directly with a mutant lacking a functional CCM. This comparison provides a direct test of whether CCM function influences the response of ϵ_p to irradiance. Recent measurements of Rubisco KIEs across β -cyanobacterial lineages also provide an independent benchmark for evaluating maximum ϵ_p . Here, we combine previously published high-irradiance measurements with new low-irradiance measurements of ϵ_p in wild-type and Δccm strains of *Synechococcus* 7002 grown under multiple CO₂ conditions. We test whether β -cyanobacterial ϵ_p responds to carbon supply and demand as predicted by the traditional flux-balance framework, whether its response to irradiance depends on CCM function, and whether maximum ϵ_p approaches the independently constrained KIE of β -cyanobacterial Rubisco.

3. Methods

3.1. Culturing

Dilute batch culture experiments were conducted using *Synechococcus* sp. strain PCC 7002 (*Synechococcus* 7002) and a previously engineered Δccm mutant strain, in which the CCM operon containing the majority of carboxysome-associated genes was knocked out, resulting in a high-CO₂-requiring phenotype (Hill et al., 2020). Cultures were grown in A+ growth medium (Stevens Jr. et al., 1973) at 37°C under continuous illumination. High- and low-irradiance treatments corresponded to photosynthetically active radiation (PAR) of $\sim 227 \pm 5 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ or $\sim 11 \pm 1 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, respectively. Irradiance was provided by cool-white fluorescent lamps and measured by a light meter (LI-COR LI-250A). Cultures were grown in 125 mL conical flasks with foam stoppers (Jaece Industries Identi-plugTM), continuously shaking, in Percival AL-41L4 incubators that kept headspace CO₂ constant by continuous replacement with a mixture of CO₂ and air during each experiment. Headspace CO₂ varied across three experimental conditions: 0.04% (v/v) CO₂ (air), 1% (v/v) CO₂, and 3% (v/v) CO₂.

Strains were acclimated to each CO₂ condition through four consecutive serial transfers. Based on growth curves collected under each experimental condition, we identified an OD₇₃₀ of 0.2 as the threshold at which cultures transitioned from exponential to linear growth and harvested cultures before they reached this

threshold. Each acclimation was inoculated with 1-3% of the final cell density and grown to an OD_{730 nm} of ~0.2 before inoculating the next culture and harvesting biomass. Harvested biomass was pelleted via centrifugation and stored at -70°C. Growth rates were calculated from the initial and final time points using:

$$\mu = \frac{\ln(\text{cell density}_{\text{final}}) - \ln(\text{cell density}_{\text{initial}})}{(t_{\text{final}} - t_{\text{initial}})}$$

where ‘ μ ’ is the specific growth rate and ‘ t ’ is the time at initial and final time points.

The high irradiance culture growth and biomass isotope measurements were reported previously by Hurley et al. (2021). For the present analysis, we included only biological replicates from transfers 3 and 4, after cultures had undergone at least two preceding transfers under the experimental conditions. Measurements from transfers 1 and 2 were excluded because the cultures were still acclimating. The low irradiance experiments and all comparisons between irradiance treatments are reported here for the first time. Because the Δccm mutant was previously shown not to grow in air or 0.5% CO₂ under high irradiance (Hurley et al., 2021), an ambient-air treatment was not included for this strain in the low-irradiance experiment. The Δccm low-irradiance treatment at 3% CO₂ was not conducted.

3.2. Carbonate chemistry

A chemical model (PHREEQC, Version 3) was used to calculate the concentration and speciation of dissolved inorganic carbon in the culture medium over the course of the experiment (Thorstenson and Parkhurst, 2004). Model inputs were the headspace CO₂ partial pressure imposed by the incubator for each condition and the full chemical recipe for A+ growth medium used to estimate media pH/alkalinity. We used the MINTEQ (v4) database for chemical constants and added a chemical definition for Tris buffer using dissociation data from the PubChem entry for Tris buffer (CID 6503, n = 36, accessed 1/15/26). The model assumed equilibration of the headspace with the growth medium to calculate the initial DIC concentrations and speciation. We compared the calculated pH values of the media to measured pH values (Figure S1) and found good agreement ($R^2 = 0.93$, $p < 0.001$). Calculated [CO₂], [HCO₃⁻], total DIC, and pH are found in Table 1.

We also accounted for the culture altering initial DIC conditions via CO₂ uptake in the following manner: The relationship between cell density and pH was characterized over relevant cell densities underneath the ~0.2 OD_{730 nm} threshold. We used calculated conditions at this upper pH threshold (i.e. harvest) as well as initial conditions as boundaries of the DIC levels experienced by the cultures over the course of the experiments. The range of aqueous CO₂ concentrations is indicated as horizontal error bars in Figure 2.

3.3. Carbon isotope analyses

Biomass samples were pipetted into tin capsules for analysis. The carbon isotopic composition of biomass was determined by first combusting samples in a Thermo Scientific FlashEA under a flow of He gas. The resultant CO₂ was analyzed with a Thermo Scientific Delta V Isotope Ratio Mass Spectrometer in continuous-flow mode. Carbon isotope compositions are expressed as the relative per mil (‰) difference between the ratio of ¹³C-¹²C in the sample (¹³C/¹²C_{sample}) and a standard of Vienna Pee Dee Belemnite (¹³C/¹²C_{VPDB}) according to $\delta^{13}\text{C} = [({}^{13}\text{C}/{}^{12}\text{C}_{\text{sample}}/{}^{13}\text{C}/{}^{12}\text{C}_{\text{standard}}) - 1] * 1000$ (‰). Acetanilide (University of Indiana; $\delta^{13}\text{C}$ of -29.53‰; weight % C of 71.09) was used to correct for linearity and drift. Acetanilide was

207 additionally used as a monitoring standard and a bovine gelatin (puget), from University of California Santa
208 Cruz ($\delta^{13}\text{C} = -12.62\text{‰}$; weight % C = 44.02) as a discrimination standard.

209
210 The carbon isotopic composition of headspace CO_2 was measured in the Earth Systems Stable Isotope
211 Laboratory at the University of Colorado Boulder using a Thermo Scientific 253 Plus isotope ratio mass
212 spectrometer. Each gas sample was analyzed in four runs, each consisting of seven cycles measured at 12.5
213 V on m/z 44 with an integration time of 26 s per cycle. Carbon isotope compositions are reported in per mil
214 (‰) relative to VPDB. Samples were measured against an in-house reference gas calibrated using
215 commercially obtained Oztech reference gases spanning a range of isotopic compositions. In Hurley et al.
216 (2021), $\delta^{13}\text{CO}_2$ for the high irradiance ambient-air treatment was estimated from atmospheric monitoring
217 data because a direct measurement was not then available. The ambient headspace was subsequently
218 measured, and the directly measured $\delta^{13}\text{CO}_2$ value is used in the present analysis.

219 The isotopic composition of dissolved CO_2 was calculated from measured headspace $\delta^{13}\text{C}$ values using the
220 temperature-dependent equilibrium fractionation between gaseous and dissolved CO_2 (Zhang et al., 1995;
221 Zeebe and Wolf-Gladrow, 2001). At 37°C, dissolved CO_2 was calculated to be approximately 1.13‰
222 depleted in ^{13}C relative to headspace CO_2 . Net photosynthetic carbon isotope fractionation was calculated
223 as:

$$\varepsilon_p = 1000 \times \left[\frac{\delta^{13}\text{C}_{\text{CO}_2(\text{aq})} + 1000}{\delta^{13}\text{C}_{\text{biomass}} + 1000} \right] - 1$$

225 where $\delta^{13}\text{C}_{\text{CO}_2(\text{aq})}$ and $\delta^{13}\text{C}_{\text{biomass}}$ are the carbon isotopic compositions of dissolved CO_2 and biomass,
226 respectively.

227 Carbonate chemistry, dissolved CO_2 isotope compositions, and ε_p were calculated consistently for the
228 previously published high irradiance measurements and the new low irradiance measurements using the
229 procedures described above.

230 3.4. Statistical analyses

231
232 Statistical analyses were conducted separately for wild-type and Δccm strains because the full irradiance–
233 CO_2 matrix was not available for the Δccm mutant. Wild-type ε_p and specific growth rates were analyzed
234 using two-factor linear models with irradiance, CO_2 condition, and their interaction as fixed effects.
235 Residual normality and homogeneity of variance were evaluated using Shapiro–Wilk and Levene’s tests,
236 respectively. The ε_p model met both assumptions, and pairwise comparisons among the six treatment means
237 were Tukey-adjusted. Growth-rate variances differed among conditions and residuals departed from
238 normality, so growth-rate effects were evaluated using heteroscedasticity-consistent (HC3) standard errors,
239 with pairwise comparisons Holm-adjusted within each set of comparisons (CO_2 within irradiance;
240 irradiance within CO_2). Prespecified comparisons within the incomplete Δccm design and between strains
241 under matched conditions were evaluated using Welch’s t tests, with Holm correction applied separately to
242 each set. Statistical significance was evaluated at $\alpha = 0.05$.

4. Results

To determine how CO₂ concentration, irradiance, and carbon-concentrating mechanism (CCM) function affect carbon isotope fractionation, we analyzed wild-type *Synechococcus* 7002 and a Δccm mutant across two irradiance levels and three CO₂ conditions (Table 1). The dataset combines previously published high-irradiance measurements with new low-irradiance measurements.

4.1. Carbonate chemistry

Calculated values for aqueous CO₂ increased linearly with headspace $p\text{CO}_2$ as expected while HCO₃⁻ and total DIC have a non-linear relationship due to pH effects on ion speciation (Table 1). We found good agreement between calculated and measured pH values (Figure S1, $R^2 = 0.93$, $p < 0.001$). Most importantly, we calculated that treatments were fully separable in CO₂ concentration as culture growth was not high enough ($\text{OD}_{730} < 0.2$) to cause overlap of CO₂ or pH conditions.

Table 1. Experimental conditions and isotopic data for wild-type *Synechococcus* 7002 (WT) and a mutant lacking a carbon-concentrating mechanism (Δccm). DIC, HCO₃⁻, CO₂, and pH are modeled initial values. Headspace CO₂ was imposed by the incubator. Isotopic parameters are reported as the mean and standard deviation of at least four biological replicates (n). High-irradiance culture growth and biomass isotope measurements were reported previously by Hurley et al. (2021) and were reanalyzed here using acclimated cultures from transfers 3 and 4, the subsequently measured $\delta^{13}\text{C}_{\text{CO}_2}$ of the ambient-air headspace, and the calculations described in Methods. Low-irradiance measurements and all comparisons between irradiance treatments are new. An ambient-air treatment was not included for the Δccm mutant because it was previously shown not to grow in air or 0.5% CO₂ (Hurley et al., 2021). The Δccm low-irradiance treatment at 3% CO₂ was not conducted.

n	PAR ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)	Headspace CO ₂ (%)	DIC ($\mu\text{mol kg}^{-1}$)	HCO ₃ ⁻ ($\mu\text{mol kg}^{-1}$)	CO ₂ ($\mu\text{mol kg}^{-1}$)	pH	μ (h ⁻¹) [†]	$\delta^{13}\text{C}_{\text{CO}_2}$ (‰) [†]	$\delta^{13}\text{C}_{\text{biomass}}$ (‰) [†]	ϵ_p (‰) [†]
WT										
5	11	0.04	521	495	8	7.9	0.03 ± 0.00	-11.0 ± 0.0	-32.4 ± 0.3	21.3 ± 0.3
6	11	1%	2758	2537	202	7.2	0.03 ± 0.00	-46.3 ± 0.0	-64.9 ± 0.4	18.6 ± 0.4
6	11	3%	3741	3127	605	6.8	0.03 ± 0.00	-48.9 ± 0.3	-67.6 ± 0.7	18.7 ± 0.7
4	227	0.04	521	495	8	7.9	0.13 ± 0.03	-11.0 ± 0.0	-19.4 ± 0.7	8.4 ± 0.7
4	227	1%	2758	2537	202	7.2	0.20 ± 0.03	-28.8 ± 0.0	-50.8 ± 0.7	22.0 ± 0.7
4	227	3%	3741	3127	605	6.8	0.15 ± 0.04	-41.7 ± 0.0	-64.1 ± 0.4	22.4 ± 0.4
Δccm										
6	11	1%	2758	2537	202	7.2	0.03 ± 0.00	-46.3 ± 0.0	-65.7 ± 0.2	19.4 ± 0.2
4	227	1%	2758	2537	202	7.2	0.14 ± 0.05	-28.8 ± 0.0	-48.2 ± 0.5	19.4 ± 0.5
4	227	3%	3741	3127	605	6.8	0.23 ± 0.01	-41.7 ± 0.0	-62.7 ± 0.2	21.0 ± 0.2

[†]Values are mean ± 1 SD (n = biological replicates).

4.2. Specific growth rates

Specific growth rates in wild-type *Synechococcus* 7002 showed an interaction between irradiance and CO₂ availability (heteroscedasticity-robust $F_{2,23} = 5.15$, $p = 0.014$). Growth rates were higher under high irradiance than under low irradiance across all three CO₂ conditions (Holm-adjusted $p < 0.001$; Figure 2A). Under low irradiance, growth rate did not differ significantly among CO₂ conditions (all adjusted $p \geq 0.277$). Under high irradiance, growth increased from low to intermediate CO₂ (adjusted $p = 0.011$). Mean growth subsequently declined from intermediate to high CO₂, although this difference was not statistically significant (adjusted $p = 0.142$).

In the Δccm mutant, growth rate was higher under high irradiance than under low irradiance at intermediate CO_2 (difference = 0.114 h^{-1} , Holm-adjusted $p = 0.031$; Figure 2B). Under high irradiance, growth also increased from intermediate to high CO_2 (difference = 0.087 h^{-1} , adjusted $p = 0.031$). The irradiance response at intermediate CO_2 did not differ significantly between strains in the robust interaction test ($F_{1,16} = 3.60$, $p = 0.076$), whereas the CO_2 response under high irradiance differed between strains ($F_{1,12} = 13.09$, $p = 0.004$). Direct growth-rate comparisons between strains under the three shared conditions were not significant after Holm correction (all adjusted $p \geq 0.069$). Maximum observed growth rates were similar in the wild type and Δccm mutant (0.20 and 0.23 h^{-1} , respectively) but occurred at intermediate and high CO_2 , respectively.

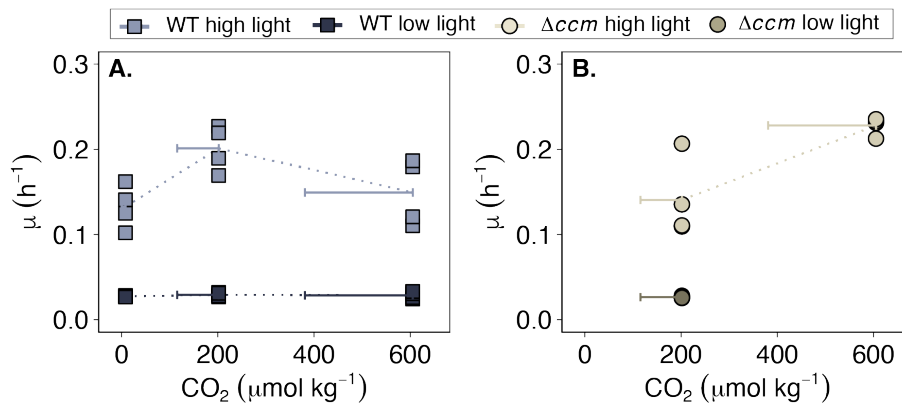


Figure 2. Specific growth rates of *Synechococcus* 7002 as a function of irradiance and dissolved CO_2 concentration. **A.** Wild-type growth rates under high irradiance (light blue symbols) and low irradiance (dark blue symbols). **B.** Growth rates of the Δccm mutant under high irradiance (light brown symbols) and low irradiance (dark brown symbols). Symbols are plotted against initial dissolved CO_2 concentrations, and horizontal error bars indicate the modeled change in dissolved CO_2 over the course of culture growth. High irradiance growth measurements were reported previously by Hurley et al. (2021) and reanalyzed as described in Methods. Low irradiance measurements and comparisons between irradiance treatments are new.

4.3. Net carbon isotope fractionation

Net carbon isotope fractionation (ϵ_p) spanned approximately 14‰ in wild-type *Synechococcus* 7002 across the experimental matrix (Table 1). The response of ϵ_p to CO_2 depended strongly on irradiance ($\text{CO}_2 \times$ irradiance interaction: $F_{2,23} = 651.75$, $p < 0.001$). Under high irradiance, ϵ_p increased by approximately 13.6‰ over an approximately 25-fold increase in dissolved CO_2 , from 8.4‰ at low CO_2 to 22.0‰ at intermediate CO_2 , and remained nearly constant at high CO_2 (22.4‰; Figure 3). Increasing CO_2 from intermediate to high concentrations did not significantly affect ϵ_p under high irradiance (difference = 0.38‰ , Tukey-adjusted $p = 0.927$). Under low irradiance, ϵ_p decreased from approximately 21.3‰ at low CO_2 to 18.6‰ at intermediate CO_2 and remained nearly constant at high CO_2 (18.7‰); the difference between intermediate and high CO_2 was not significant (difference = 0.10‰ , adjusted $p > 0.999$). Increasing

irradiance decreased ϵ_p by approximately 12.9‰ at low CO₂ but increased ϵ_p by approximately 3.4‰ and 3.7‰ at intermediate and high CO₂, respectively (all adjusted $p < 0.001$).

Isotope data for the Δccm mutant were available at intermediate CO₂ (1%) under both irradiance levels and at high CO₂ (3%) under high irradiance. Across these three treatments, ϵ_p spanned only 1.6‰ (Table 1). At intermediate CO₂, ϵ_p was approximately 19.4‰ under both irradiance levels (Holm-adjusted $p = 0.984$; Figure 3). Under high irradiance, ϵ_p increased from 19.4‰ at intermediate CO₂ to 21.0‰ at high CO₂ (difference = 1.58‰, adjusted $p = 0.007$). The irradiance response at intermediate CO₂ differed between strains (heteroscedasticity-robust $F_{1,16} = 41.42$, $p < 0.001$), whereas the strain-by-CO₂ interaction under high irradiance did not meet the threshold for significance in the robust analysis ($F_{1,12} = 4.70$, $p = 0.051$). Compared with the wild type, Δccm ϵ_p was approximately 0.8‰ higher under low irradiance at intermediate CO₂, 2.6‰ lower under high irradiance at intermediate CO₂, and 1.4‰ lower under high irradiance at high CO₂ (all Holm-adjusted $p = 0.005$). Thus, loss of CCM function substantially reduced the irradiance response of ϵ_p at intermediate CO₂, while a smaller response to CO₂ remained under high irradiance.

Across experimental-condition means, ϵ_p was not significantly related to specific growth rate in either the wild-type strain ($R^2 < 0.001$, $F_{1,4} < 0.01$, $p = 0.993$) or the Δccm mutant ($R^2 = 0.68$, $F_{1,1} = 2.11$, $p = 0.384$; Figure S2). The Δccm regression was based on only three experimental conditions and should therefore be interpreted cautiously. Variation in growth rate alone did not account for the ϵ_p responses observed across the experimental matrix.

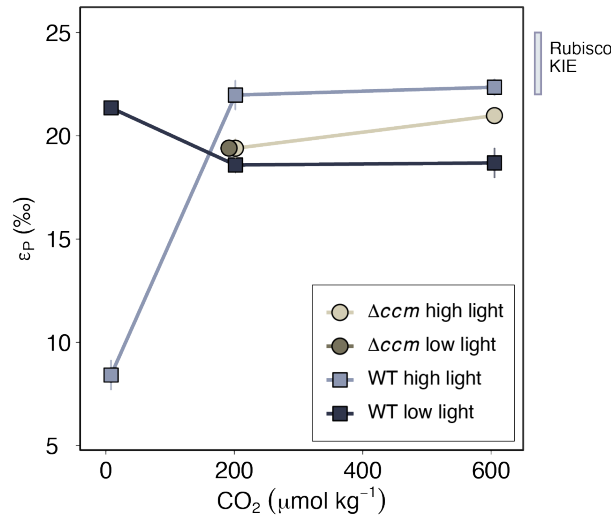


Figure 3. The relationship between ϵ_p and dissolved CO₂ concentration in wild-type *Synechococcus* 7002 (blue squares) and the Δccm mutant (brown circles) under high and low irradiance conditions. Symbols represent the means of at least four biological replicates, and vertical error bars represent standard deviations among replicates. Shaded rectangles indicate the range of kinetic isotope effects measured for Form IB Rubisco from β -cyanobacteria (Guy et al., 1993; Wijker et al., 2026; Aguiló-Nicolau et al., 2026). High irradiance culture and biomass isotope measurements were reported previously by Hurley et al. (2021) and were reanalyzed as described in Methods. Low irradiance measurements and comparisons between irradiance treatments are new.

5. Discussion

Our results show that carbon isotope fractionation in *Synechococcus* 7002 cannot be described by a single response to CO₂ availability. Across the reanalyzed high irradiance measurements and new low irradiance measurements, wild-type ϵ_p varied by approximately 14‰, demonstrating that β -cyanobacteria can express a much wider range of fractionation than previously observed in α -cyanobacteria (Popp et al., 1998). Consistent with Hurley et al. (2021), ϵ_p increased with CO₂ availability under high irradiance. Under low irradiance, however, ϵ_p decreased as CO₂ increased, revealing that irradiance can alter not only the magnitude but also the direction of the ϵ_p response to CO₂. Disruption of the CCM reduced the range of ϵ_p to approximately 2‰ and eliminated its detectable response to irradiance, providing direct evidence that CCM function contributes to the sensitivity of ϵ_p to irradiance and CO₂. Maximum ϵ_p values in both strains approached the expected KIE of β -cyanobacterial Form IB Rubisco (Figure 3). Thus, although physiological processes in addition to Rubisco carboxylation may contribute to net fractionation, their associated isotope effects are not required to explain the magnitude of the maximum ϵ_p observed here.

5.1. Conditional limitation by light and CO₂

Using batch cultures to determine how environmental and physiological factors influence ϵ_p can be challenging because resource availability and growth rate are interdependent (Burkhardt et al., 1999). Growth rate responds to the availability of light, inorganic carbon, and nutrients, but their relative influence is not always clear. Recent interpretations have invoked light limitation across nutrient-replete batch cultures to explain the influence of irradiance on ϵ_p (Wilkes and Pearson, 2019; Pearson et al., 2025). However, our results suggest that growth rate in batch culture can be constrained by either light or carbon availability.

We used growth responses to increased irradiance or CO₂ availability to infer light or carbon limitation of specific growth rate (i.e., Blackman limitation; Cullen, 1991; Moore et al., 2013). In the wild-type strain, growth rates were substantially higher at high irradiance than at low irradiance across all CO₂ treatments, consistent with light limitation under low irradiance (Figure 2). Growth rates under low irradiance did not increase with CO₂ availability, providing further evidence that light, rather than carbon supply, constrained growth rates under these conditions. Under high irradiance, however, growth rates increased from the low to the intermediate CO₂ treatment (Figure 2), indicating that carbon availability constrained growth rates at low CO₂. The growth rate under high irradiance at intermediate CO₂ was similar to the reported maximum for *Synechococcus* 7002 under optimal conditions ($\mu = 0.28 \text{ h}^{-1}$) (Bernstein et al., 2016), suggesting that this treatment supported near-maximal growth. Growth subsequently declined under high CO₂ at high irradiance, indicating that additional CO₂ did not stimulate growth and may instead have imposed another physiological constraint.

The Δccm mutant also grew faster at high irradiance than at low irradiance under intermediate CO₂ (Figure 2). In contrast to the wild-type strain, growth rates for the Δccm mutant increased from the intermediate to the high CO₂ treatment under high irradiance, approaching the reported maximum for *Synechococcus* 7002 under optimal conditions (Bernstein et al., 2016). These responses suggest that intermediate CO₂ supported near-maximal growth in the wild-type strain but still constrained growth in the Δccm mutant, consistent with the CCM supporting growth at lower external CO₂ concentrations. Together, these results suggest that light constrained growth rates at low irradiance, whereas carbon availability constrained growth over

different CO₂ ranges in the two strains at high irradiance. These inferences provide a basis for interpreting the corresponding ϵ_p responses.

5.2. Comparison to the expectations from the flux-balance framework

5.2.1. The largest ϵ_p responses follow the flux-balance framework

The wild-type ϵ_p responses were consistent with the flux-balance framework under some, but not all, experimental conditions. Under high irradiance, a 25-fold increase in dissolved CO₂ from the low to the intermediate CO₂ condition was accompanied by an approximately 13.6‰ increase in ϵ_p despite a 1.5-fold increase in growth rate (Figures 2–3). At low CO₂, a 20-fold decrease in irradiance was accompanied by a 4.3-fold decrease in growth rate and an approximately 12.9‰ increase in ϵ_p (Figures 2–3). Thus, increasing carbon supply in the first case and reducing carbon demand in the second both decreased μ/CO_2 and increased ϵ_p , producing the negative relationship expected from the flux-balance framework (Figure S2B). From the intermediate to the high CO₂ condition, higher CO₂ and lower growth rate further decreased μ/CO_2 , but ϵ_p remained approximately constant, suggesting that fractionation was approaching its maximum. The responses consistent with the flux-balance framework were also the largest changes in ϵ_p observed across the experimental matrix.

5.2.2 Maximum ϵ_p approaches Rubisco isotope discrimination

The magnitude of the maximum ϵ_p provides a second test of the flux-balance framework. Within the steady-state framework, ϵ_p approaches the kinetic isotope effect of Rubisco when carbon supply is high relative to cellular demand and a large proportion of incoming inorganic carbon escapes fixation (Rau et al., 1992; François et al., 1993; Hayes, 1993; Goerick et al., 1994). This theoretical expectation provides a basis for comparing the maximum ϵ_p observed in our experiments with independently measured kinetic isotope effects of β -cyanobacterial Rubiscos (Hayes, 1993; Laws et al., 1995).

Although the kinetic isotope effect (KIE) of Form IB Rubisco from *Synechococcus* 7002 has not been measured directly, values measured in other β -cyanobacteria range from 22.0 to 27.3‰ (Guy et al., 1993; Wijker et al., 2026; Aguiló-Nicolau et al., 2026). Among the β -cyanobacteria with measured Rubisco KIEs, *Cyanobacterium aponinum* PCC 10605 is the closest phylogenetic relative of *Synechococcus* 7002 (Strunecký et al., 2023) and has a Rubisco KIE of $25.2 \pm 0.4\text{‰}$ (Aguiló-Nicolau et al., 2026). The CO₂/O₂ specificity of *Synechococcus* 7002 Rubisco ($S_{\text{C/O}} = 52$) (Andrews, 1988; Tcherkez et al., 2006) lies between those of *C. aponinum* ($S_{\text{C/O}} = 58.6 \pm 2.1$) (Aguiló-Nicolau et al., 2026) and *Synechococcus* sp. PCC 6301 ($S_{\text{C/O}} = 48.2 \pm 5.8$) (Aguiló-Nicolau et al., 2023). Rubisco KIE values measured for *Synechococcus* sp. PCC 6301 are approximately 22–23‰ (Guy et al., 1993; Wijker et al., 2026). Given the theoretically predicted positive relationship between $S_{\text{C/O}}$ and Rubisco KIE (Tcherkez et al., 2006) and the empirical relationship observed within cyanobacteria (Aguiló-Nicolau et al., 2026), the KIE of *Synechococcus* 7002 Rubisco may fall within the approximately 22–25‰ range bounded by these measurements.

The maximum ϵ_p observed in the wild-type strain, approximately 22.4‰, fell near the lower end of this inferred Rubisco KIE range, while the maximum of approximately 21.0‰ in the Δccm mutant approached the lower limit. While it remains possible that additional physiological fractionations, including those proposed for vectorial carbonic anhydrase activity associated with NDH-1 CO₂-uptake complexes (Wilkes and Pearson, 2019; Wang et al., 2023) contributed to variation in ϵ_p among treatments, they are not necessarily required to explain the maximum ϵ_p observed here.

5.2.3 ϵ_p increases with irradiance at elevated CO₂

Although the maximum ϵ_p was consistent with the inferred Rubisco KIE, the flux-balance framework did not explain the full pattern of ϵ_p across the experimental matrix. At intermediate and high CO₂, a 20-fold increase in irradiance was associated with increases in ϵ_p of approximately 3.4‰ and 3.7‰, respectively, despite approximately 5–7-fold higher growth rates (Figures 2–3). These responses are opposite to the flux-balance prediction: faster growth at higher irradiance should increase carbon demand relative to supply and decrease ϵ_p .

A similar positive relationship between ϵ_p and irradiance has been observed in other phytoplankton, including the haptophyte alga *Gephyrocapsa huxleyi* (*G. huxleyi*). In *G. huxleyi*, ϵ_p increases by approximately 5–7‰ across irradiances of 12–300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Thompson and Calvert, 1995; Rost et al., 2002; Trimborn et al., 2007). Chemostat experiments show that ϵ_p increases with irradiance when growth rate is controlled (Pearson et al., 2025) but decreases with growth rate when irradiance is held constant (Bidigare et al., 1997). These opposing responses may partially offset one another (Pearson et al., 2025), particularly in batch cultures, where increasing irradiance can also increase growth rate. The similar increase in ϵ_p at high irradiance observed in *Synechococcus* 7002 may therefore reflect a comparable light-dependent mechanism. In our experiments this response occurred at elevated CO₂, where carbon availability did not appear to constrain wild-type growth rates, partially analogous to nutrient-limited chemostat experiments in which growth rate is constrained by nutrient rather than carbon availability (Pearson et al., 2025).

Comparison with the Δccm mutant provides a way to evaluate whether the irradiance response of ϵ_p was associated with CCM function in *Synechococcus* 7002. At intermediate CO₂, wild-type ϵ_p was approximately 3.4‰ higher under high irradiance than under low irradiance, whereas ϵ_p in the Δccm mutant was approximately 19.4‰ under both irradiance conditions (Figure 3). The absence of a detectable irradiance response in the mutant provides strong evidence that the positive ϵ_p response to irradiance in the wild type depends on CCM function.

Irradiance can affect CCM operation by supplying the photosynthetic energy required for active inorganic carbon acquisition and by regulating CCM components (Badger et al., 2006; Ludwig and Bryant, 2011). In cells grown at 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and 1% CO₂, transfer to darkness for one hour reduced transcripts encoding Rubisco, carboxysome components, and carboxysomal carbonic anhydrase, whereas transcripts for the SbtA and BicA bicarbonate transporters remained approximately unchanged (Ludwig and Bryant, 2011). These acute responses do not reproduce acclimation to the irradiance levels used in our experiments, but they show that light availability affects specific components of carbon uptake, concentration, and fixation differently.

The higher ϵ_p observed under high irradiance is directionally consistent with three possibilities: greater use of CO₂ relative to HCO₃[−], loss of a larger fraction of acquired inorganic carbon through leakage, or expression of an additional isotope effect associated with CCM function. Greater use of CO₂ relative to HCO₃[−] would increase ϵ_p because CO₂ is depleted in ¹³C relative to HCO₃[−] at isotopic equilibrium (Mook et al., 1974). However, there is no clear physiological reason to expect greater reliance on CO₂ under high irradiance. *Synechococcus* 7002 uses Na⁺ dependent transporters SbtA and BicA to accumulate HCO₃[−], and

greater photosynthetic energy availability could instead support active HCO_3^- acquisition (Price et al., 2004; Woodger et al., 2007). A shift toward greater CO_2 use is therefore possible but is not expected from energy availability alone.

Greater fractional leakage would allow more complete expression of the Rubisco KIE and increase ϵ_p . Light dependent CO_2 efflux and inorganic carbon cycling have been observed directly in other cyanobacteria (Tchernov et al., 1997; de Araujo et al., 2011), providing a physiological basis for leakage to vary with irradiance. The transcriptional responses reported by Ludwig and Bryant (2011) further suggest that irradiance can alter inorganic carbon uptake, intracellular concentration, and fixation, all of which influence fractional leakage. However, increased CO_2 efflux would increase fractional leakage only if carbon loss increased relative to inorganic carbon acquisition and fixation. Irradiance can affect each of these processes, so these physiological responses do not establish whether fractional leakage increases with irradiance in *Synechococcus* 7002.

Finally, NDH-1 CO_2 uptake complexes couple photosynthetic electron transport to vectorial CO_2 hydration, a reaction proposed to introduce an additional isotope effect (Wilkes and Pearson, 2019; Wang et al., 2023). If this reaction contributes to net carbon isotope fractionation, greater activity under high irradiance could increase ϵ_p independently of changes in the Rubisco KIE or fractional leakage. In *Synechococcus* 7002, *ndhD3* and *ndhF3* transcripts were approximately 2.5 to 4 times more abundant under standard illumination at $250 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ than after one hour of darkness, whereas *cupA* and *cupS* transcripts changed to a lesser degree (Ludwig and Bryant, 2011). This partial response is consistent with light sensitivity of the inducible NDH-1 complex but does not establish a change in vectorial CO_2 hydration. The positive response of ϵ_p to irradiance at elevated CO_2 and the absence of this response in the Δccm mutant are also directionally consistent with the hypothesis. However, the mutant comparison implicates CCM function broadly rather than the NDH-1 complexes specifically. Thus, although the Δccm comparison associates the irradiance response with CCM function, the available physiological evidence does not identify the mechanism.

5.2.4 ϵ_p decreases with CO_2 under low irradiance

A second departure from the flux-balance framework occurred in the wild-type strain under low irradiance. Growth rates remained low and relatively constant across CO_2 treatments (Figure 2), suggesting little change in growth-related carbon demand. Increasing CO_2 availability would therefore be expected to increase carbon supply relative to demand and produce higher ϵ_p . Instead, ϵ_p decreased by approximately 2.7‰ between the low and intermediate CO_2 treatments, from 21.3‰ to 18.6‰, and remained approximately constant at high CO_2 (Figure 3). This decrease is opposite to the response expected from increasing external CO_2 availability at a nearly constant growth rate.

The high ϵ_p observed under low irradiance and low CO_2 is not itself inconsistent with the flux-balance framework. Wild-type *Synechococcus* 7002 produced similarly high ϵ_p under two contrasting conditions: high irradiance with elevated CO_2 and low irradiance with low CO_2 . Within the framework, these conditions could produce high ϵ_p through different routes to a low carbon demand-to-supply ratio: greater carbon supply under elevated CO_2 or lower carbon demand under low irradiance. What remains unexplained is the subsequent decrease in ϵ_p as CO_2 availability increased under low irradiance. Limited photosynthetic energy could have reduced active HCO_3^- uptake and increased reliance on CO_2 , but this shift would be expected

to increase rather than decrease ϵ_p (Mook et al., 1974). Thus, neither the flux-balance framework nor a simple change in the relative use of CO_2 and HCO_3^- explains the observed decline.

5.2.5 Systematic patterns in the ϵ_p response

Collectively, these results reveal three systematic features of the ϵ_p response. First, the largest changes in ϵ_p occurred when the availability of the factor constraining growth changed. Increasing CO_2 when carbon availability constrained growth increased ϵ_p by approximately 13.6%, whereas increasing irradiance when light constrained growth decreased ϵ_p by approximately 12.9%. Although these responses were driven by different environmental factors, both altered the balance between carbon supply and demand in the direction expected by the flux-balance framework. Second, irradiance affected ϵ_p in ways that were both consistent with and contrary to the framework. Its effect at low CO_2 was consistent with the accompanying change in growth rate, whereas at elevated CO_2 , ϵ_p increased with irradiance despite faster growth. Third, the absence of an observed irradiance response in the Δccm mutant supports a role for CCM function in the positive irradiance response of the wild-type strain. Thus, the flux-balance framework captured the direction of the largest ϵ_p responses, while the smaller but systematic departures show that irradiance and CCM function also affect ϵ_p through processes not represented in the framework.

5.3 Environmental and proxy implications

The contrasting ϵ_p ranges of α - and β -cyanobacteria raise the question of whether their responses reflect adaptation to different carbon regimes. Open-ocean α -cyanobacteria generally experience relatively stable carbonate chemistry, whereas many β -cyanobacteria occur in environments such as dense blooms, mats, and coastal waters where inorganic carbon availability can vary widely and carbon limitation may occur more frequently (Badger et al., 2006). The abundance of α -cyanobacteria in large freshwater lakes further suggests that environmental stability, rather than salinity itself, may be the relevant ecological distinction (Cabello-Yeves et al., 2022). If the contrasting ϵ_p ranges of α - and β -cyanobacteria reflect adaptation to environments with different degrees of carbon variability and limitation, their isotopic signatures may contain information about ecological setting as well as ambient CO_2 . Variation in cyanobacterial community composition could therefore contribute to variability in sedimentary ϵ_p that is not directly related to atmospheric CO_2 .

The relative importance of factors known to control ϵ_p in phytoplankton varies across marine environments. Previous culture studies have shown that irradiance can affect ϵ_p in ways that are not explained by its influence on growth rate alone (Thompson and Calvert, 1995; Rost et al., 2002; Trimborn et al., 2007; Pearson et al., 2025). Our results extend this pattern to a β -cyanobacterium and systematically show that irradiance can alter the direction as well as the magnitude of the ϵ_p response to CO_2 . Despite these departures, the largest ϵ_p responses in our experiments followed the direction predicted by the flux-balance framework (Figure S2). This pattern may help explain why ϵ_p covaries with CO_2 in sedimentary records despite additional environmental and physiological controls. A broader mechanistic framework must therefore integrate CO_2 -driven changes in carbon supply and demand with irradiance-dependent physiological responses.

5.4 Expected ϵ_p responses across contrasting environments

The relative effects of light, inorganic carbon, and nutrients on phytoplankton growth rates vary across aquatic environments. Nutrient availability constrains phytoplankton growth throughout much of the

surface ocean (Browning and Moore, 2023). During deep vertical mixing, however, low mean irradiance can limit primary productivity even when nutrients remain abundant (Cullen, 1991). In well-lit, nutrient-replete environments, rapid growth and biological CO₂ drawdown can instead increase carbon demand relative to supply (Bidigare et al., 1997; Benthien et al., 2007). Our results suggest that the response of ϵ_p to CO₂ availability may differ between these light-limited and carbon-limited conditions. More broadly, the mechanisms controlling ϵ_p may vary among environments depending on the prevailing light, inorganic carbon, and nutrient regimes.

Phytoplankton blooms provide an example of an environment in which the factor constraining growth can change over time, with potential implications for ϵ_p . Following an influx of nutrients into well-lit surface waters, relief of nutrient limitation can allow growth rates to increase. As biomass accumulates, biological carbon fixation can draw down dissolved CO₂, increased light attenuation can reduce the irradiance experienced by cells, and nutrient depletion can eventually reduce growth rates. The relative influences of inorganic carbon, light, and nutrients can therefore shift through bloom development. Measurements from natural and experimental blooms show that ϵ_p also changes over the course of a bloom, decreasing during some periods of exponential growth and CO₂ drawdown and stabilizing or increasing as nutrient limitation reduces growth rates (Kukert and Riebesell, 1998; Gervais and Riebesell, 2001; Benthien et al., 2007). Our results suggest that changes in the factor constraining growth could contribute to ϵ_p variation over the course of a bloom, although the relative effects of CO₂ availability, irradiance, nutrients, and community composition remain difficult to separate.

Depth-dependent variation in irradiance may also affect the ϵ_p recorded by exported organic carbon. Particulate organic carbon is commonly depleted in ¹³C in the lower euphotic zone relative to the upper euphotic zone (Close and Henderson, 2020) and Henderson et al. (2024) measured a 5.6‰ increase in ϵ_p with depth at a site in the subtropical North Atlantic. Models based on growth rate, CO₂ availability, and cell geometry did not fully account for this vertical difference. Our results provide experimental evidence that irradiance can affect ϵ_p beyond its influence on growth rate and that the direction of this effect depends on CO₂ availability. Changes in the depth and light environment of organic-carbon production could therefore alter the ϵ_p of exported carbon without a corresponding change in atmospheric CO₂.

Cyanobacterial mats provide another setting in which primary producers experience steep spatial and temporal variation in both irradiance and inorganic-carbon supply. Light attenuation, diffusion across the mat–water boundary, photosynthetic carbon fixation, and respiration produce gradients in irradiance, CO₂, O₂, and pH over millimeter scales and across diel cycles (Jensen et al., 2011; Houghton et al., 2014). In a hypersaline microbial mat, carbon fixation exceeded diffusive CO₂ supply in the upper photic zone, producing local CO₂ limitation, whereas respiration exceeded carbon fixation below approximately 7 mm (Houghton et al., 2014). Primary production within mats therefore occurs across depth-dependent combinations of irradiance and inorganic-carbon availability. The primary carbon isotope signal produced in mats may therefore depend on where and when carbon fixation and subsequent carbon processing occur within these gradients.

5.5 Implications for sedimentary carbon-isotope records

Sedimentary carbon isotope records integrate organic carbon produced under different irradiance, nutrient, and inorganic carbon regimes. In traditional pCO₂ reconstructions, aqueous CO₂ is estimated from ϵ_p using

a physiological parameter, b , that incorporates the effects of growth rate, cell geometry, and other physiological factors on fractionation (Freeman and Pagani, 2005). In *Synechococcus* 7002, the relationship between ϵ_p and CO_2 differed between irradiance treatments. Under high irradiance, the change from the low to the intermediate CO_2 treatment increased ϵ_p by approximately 13.6‰, whereas under low irradiance the same treatment change decreased ϵ_p by approximately 2.7‰ while growth rate remained nearly constant. A fixed or growth based parameterization of b would not capture both responses. If similar irradiance dependence occurs in organisms contributing to pCO_2 proxy records, changes in the light environment of production could bias or obscure pCO_2 trends inferred from ϵ_p .

The implications of these results may be particularly relevant to phytane records, which integrate chlorophyll derived from the broader phytoplankton community, and to bulk organic carbon records that can include cyanobacterial organic matter (Witkowski et al., 2018; Hurley et al., 2021). However, a similar positive ϵ_p response to increasing irradiance has been observed in the alkenone producer *Gephyrocapsa huxleyi* (Thompson and Calvert, 1995; Rost et al., 2002; Trimborn et al., 2007; Pearson et al., 2025), raising the possibility that this systematic response occurs across more than one phytoplankton lineage. Whether the underlying mechanism is shared and whether the direction of the response is consistent across phytoplankton remain unresolved. Establishing the generality of irradiance dependent ϵ_p responses will be important for determining how changes in the light environment of production are expressed in different pCO_2 proxy records.

6. Conclusions

The response of ϵ_p to CO_2 in *Synechococcus* 7002 depended on irradiance and CCM function. Wild type ϵ_p varied by approximately 14‰ across the experimental matrix, increasing with CO_2 under high irradiance but decreasing with CO_2 under low irradiance. The largest ϵ_p responses occurred when the availability of the factor constraining growth changed and followed the direction predicted by the flux balance framework. However, the positive ϵ_p response to irradiance at elevated CO_2 and the negative ϵ_p response to CO_2 under low irradiance were not explained by the framework. The narrow ϵ_p range in the Δccm mutant further supports a role for CCM function in producing the environmental sensitivity observed in the wild type.

The maximum wild type ϵ_p of approximately 22.4‰ was consistent with the lower end of the 22–25‰ range inferred for *Synechococcus* 7002 Rubisco, indicating that additional physiological fractionations are not required to explain the magnitude of the observed maximum. More broadly, our results show that the flux balance framework can capture the direction of the largest ϵ_p responses without explaining the full response across irradiance and CO_2 conditions. Because irradiance altered the relationship between ϵ_p and CO_2 , determining whether this response extends across phytoplankton lineages will be important for developing mechanistic models and interpreting pCO_2 proxy records.

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Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

The authors used Codex (OpenAI) to improve the clarity and readability of the manuscript. All AI-assisted revisions were reviewed and edited by the authors, who take full responsibility for the final manuscript.

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Supplementary Material:
Light- and CO₂-dependent carbon isotope fractionation in the β -cyanobacterium *Synechococcus* sp. PCC 7002

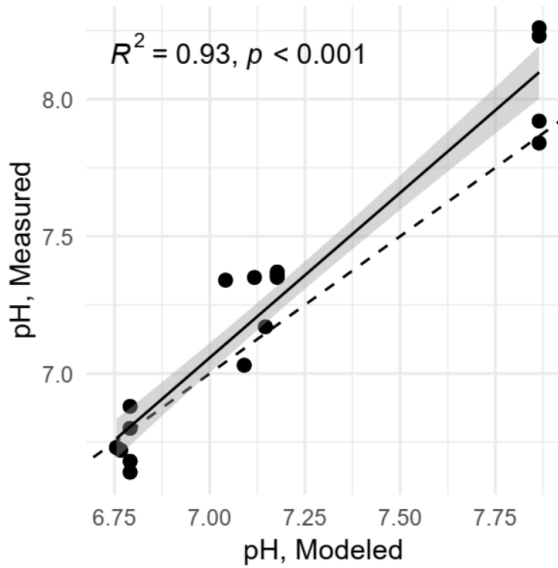


Figure S1. Comparison of calculated and measured pH values. Modeled pH values for individual culture flasks (x-axis) are compared with measured pH values (y-axis). The solid black line shows the linear fit to the data, and the shaded region represents its 95% confidence interval. The coefficient of determination (R^2) and p value are shown in the panel. The dashed line represents a 1:1 relationship between modeled and measured values.

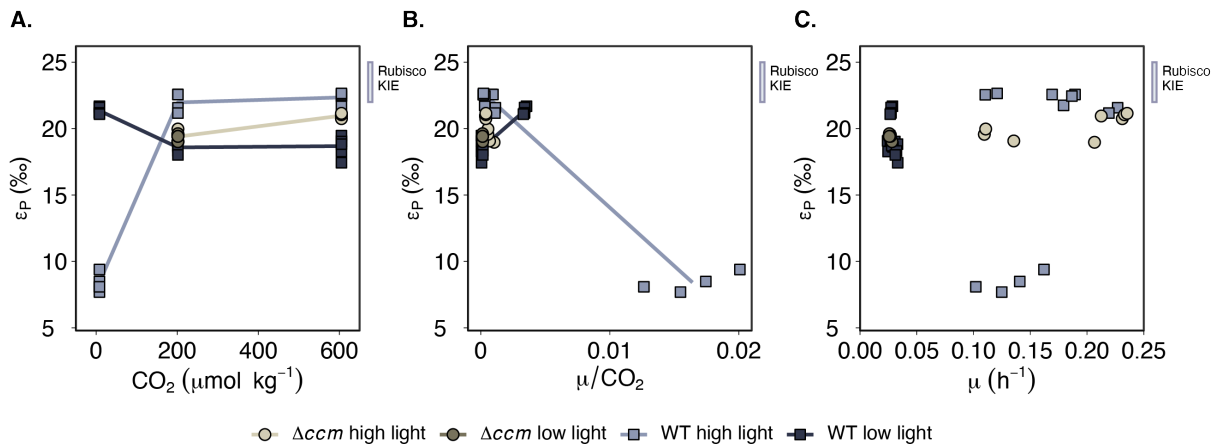


Figure S2. Values of ϵ_p for wild-type *Synechococcus* 7002 and the Δccm mutant as functions of **A.** dissolved CO₂ concentration, **B.** μ/CO_2 , and **C.** specific growth rate (μ). High and low irradiance correspond to approximately 227 and 11 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, respectively. High-irradiance culture growth and biomass isotope measurements were reported previously by Hurley et al. (2021) and were reanalyzed as described in Methods. Low-irradiance measurements and comparisons between irradiance treatments are new. For the Δccm mutant, data were available at 1% CO₂ under both irradiance levels and at 3% CO₂ under high

20 irradiance. No ambient air treatment was conducted for the *Δccm* mutant under low irradiance because the
21 mutant was previously shown not to grow in air or 0.5% CO₂ under high irradiance (Hurley et al., 2021).