Answer reviewer comments for manuscript [egusphere-2025-5010]

Comments from the reviewers are marked as "**rev {reviewer no./comment no.}**" in italicized font. The answers of the authors are indented and follow immediately after the reviewer comments. General statements made by the reviewers are not reproduced here. highlighted and ~~strikethrough~~ text shows changes in the manuscript. Line numbers (L) mentioned in our replies correspond to the original single-column (unrevised) version of the manuscript.

Reviewer 2

General comments

rev 2/1 *Your new simplified approach, consisting of semi-purified extracts and the Apollo-Picarro DIC-$\delta^{13}C$ analyzer, gives accurate results while saving time and effort. I would recommend to present this specific new methodology in the abstract. I would also recommend to introduce the approach at the end of the introduction, so that it's clear for the reader what you are going to do.*

We agree with the reviewer and have revised the manuscript accordingly. The simplified methodological approach combining semi-purified extracts with the Apollo – Picarro δ^{13}C-DIC analyzer is now explicitly described in the Abstract. In addition, we introduce this approach at the end of the Introduction to clearly outline the experimental strategy and objectives of the study for the reader. The revised part of the Abstract and Introduction are provided below.

(L8-L10): "...Here, we present a simplified method that overcomes these limitations, eliminating the need for complex purification protocols, specialized equipment, and experimental designs that yield little CO_2 fixation and high uncertainties. We use a simplified purification procedure yielding semi-purified Rubisco extracts, together with an Apollo–Picarro δ^{13}C-DIC analyzer capable of simultaneously measuring DIC concentration and ^{13}C isotope ratios. Using this protocol, we accurately determined $\epsilon_{\text{Rubisco}}$ for..."

(L80-L85): "Despite the ~~significance~~ importance of $\epsilon_{\text{Rubisco}}$ for biogeochemical and evolutionary models, no standardized and accessible protocol exists for its determination across diverse phylogenetic groups. Here, we introduce a simplified ~~propose a~~ method that overcomes ~~provides several advantages over existing approaches and enables $\epsilon_{\text{Rubisco}}$ determination~~ key limitations of existing approaches and enables robust $\epsilon_{\text{Rubisco}}$ measurements across a wide range of taxa Specifically, we couple a rapid Rubisco semi-purification method to an Apollo–Picarro δ^{13}C-DIC analyzer, avoiding the need for time-consuming full enzyme purification while enabling simultaneous quantification of DIC concentration and isotopic composition. We compare the performance of this semi-purified preparation with a more complex protocol yielding fully purified Rubisco, and we assess the utility of the Apollo–Picarro δ^{13}C-DIC analyzer relative to classical GasBench-IRMS measurements. In addition, we incorporate a simple kinetic model to account for DIC consumption dynamics during incubations, thereby providing a rational basis for selecting appropriate enzyme

concentrations and sampling intervals. Using this simplified approach, we demonstrate reproducibility in ~~, using a single instrument for simultaneous quantification of total DIC and its isotopic composition and with sample concentration and sampling times supported by a realistic kinetic model of DIC consumption by Rubisco from different taxa. We illustrate the reproducibility of the method for S.~~ *Spinacia oleracea* and *Synechococcus* sp. and provide ~~a first determination for~~ the first determination of $\epsilon_{\text{Rubisco}}$ for the coccolithophore *Gephyrocapsa oceanica*."

Specific comments

rev 2/2 *The methods section (2.2.) contains a description of the partial- and full purification method. However, at this point in the text it is not yet clear why these different methods are applied (e.g., why not only use the full purification method? See general comment). Only in the results section (3.1.) this becomes clear (fast/simple versus time-intensive). I would recommend integrating section 3.1 with methods section 2.2 for clarity.*

We agree that the rationale for applying both partial and full purification protocols should be clear at the Methods stage. Rather than restructuring and integrating Sections 2.2 and 3.1, we have expanded the final paragraph of the Introduction to explicitly describe the experimental strategy employed in this study (see **rev 2/1**). This revision explains the motivation for using both purification approaches – namely, the comparison of a rapid, simplified workflow with a more time-intensive full purification – and clarifies this rationale prior to the Methods section. We believe this change sufficiently addresses the reviewer's concern while preserving a clear separation between Methods and Results.

rev 2/3 *Furthermore, section 3.1. mentions the assessment of the degree of Rubisco purification for the two methods using SDS-PAGE. This abbreviation (SDS-PAGE) is however not yet described in the text. Therefore, I would include a short explanation of SDS-PAGE in the Methods section.*

We thank the reviewer for pointing this out. To clarify the abbreviation and methodology, we have added a new subsection (Section 2.2.3) to the Methods that briefly describes SDS–PAGE and explains how it was used to assess the degree of Rubisco purification for the semi-purified and fully purified protocols.

rev 2/4 *Lines 159 – 165 (or whole section 2.3): Can't this be integrated with section 2.4.2? It feels a bit redundant to explain the Apollo-Picarro method and GasBench method twice, in other words.*

We agree that some redundancy existed between Sections 2.3 and 2.4.2. These sections serve distinct purposes: Section 2.3 focuses on the isotope fractionation experiments and sample handling up to injection into the analytical instruments, whereas Section 2.4.2 describes the measurement procedures, calibration standards, and analytical uncertainties. We have removed redundant descriptions from both sections to avoid repetition, while keeping the methodological separation to clearly distinguish experimental execution from analytical measurement (see below).

L159-L165: "...Each sample was divided into two fractions. The first fraction, containing at least 1.25 μmol DIC (0.5-2 mL), was diluted into 2 mL of N_2-purged 110 mM EPPS buffer (pH 7.8) and either injected directly into a DIC-δ^{13}C Analyzer ~~(AS-D1 and G2131-i Apollo-Picarro, USA)~~ or filtered through a 50 kDa Amicon Ultra-4 centrifugal unit (UFC8010, Merck, USA) at 2,000 × g for 3 minutes at 25 °C before injection for concentration and carbon isotope analysis of DIC. The second fraction, containing at least 0.3 μmol DIC (0.1-0.5 mL), was immediately injected into a 5 mL septum-capped vial flushed with helium and preloaded with 0.1 mL of 200 mM H_3PO_4. These samples were analyzed the following day using a GasBench system ~~II system coupled via a ConFlow IV interface to a Delta V Plus isotope ratio mass spectrometer (Thermo Fisher Scientific, USA)~~..."

L179-L183: "Concentration and δ^{13}C composition of DIC were measured using an Apollo acidification system AS-D1 (Apollo SciTech, LLC, USA) coupled to a Picarro G2131-i cavity ring-down spectrometer (Picarro Inc., USA). ~~For each measurement, 0.5 to 2 mL of sample (containing at least 1.25 μmol DIC) was diluted in 2 mL of 110 mM EPPS buffer and transferred to an~~ Samples were injected into the acidification chamber, where 0.9 mL of 5 M phosphoric acid was added to convert DIC into CO_2 gas. The evolved CO_2 was subsequently ~~then~~ sparged and transferred to the Picarro analyzer for isotopic and concentration analysis."

rev 2/5 *Lines 214 – 215: In your case, the standard deviation of the Gaussian error propagation represents the uncertainty of the parameter ($\epsilon_{\text{Rubisco}}$) estimate. Therefore, it serves as the standard error of the parameter. Perhaps it would be informative to mention this, as this makes it clearer later in the text when you're comparing calculated $\epsilon_{\text{Rubisco}}$ values with other $\epsilon_{\text{Rubisco}}$ values from literature. It makes statements like 'statistically indistinguishable' (line 363), 'virtually identical' (line 417), or '...falls within a similar range...' (line 414) more substantive.*

We thank the reviewer for this important clarification. In the original manuscript, the treatment of uncertainties was described imprecisely: Gaussian error propagation was applied only to replicates measured on the same analytical instrument, whereas variability among replicates measured across both instruments was summarized using standard deviation, which we initially thought better reflected the observed between-instrument variability. We recognize that this mixed approach was confusing.

In the revised manuscript, we have standardized the uncertainty treatment by consistently applying Gaussian error propagation throughout. As a result, all reported uncertainties now represent standard errors of the $\epsilon_{\text{Rubisco}}$ parameter estimates, and this clarification is explicitly stated in the Methods section (see below). Although the resulting standard errors are generally smaller than those previously reported, this change does not affect the interpretation of the data or any conclusions drawn.

In addition, where appropriate, we now apply two-tailed t-tests to statistically support comparative statements. This further substantiates statements such as "statistically indistinguishable" (see also **rev 2/8**).

L214-215: "...Measurement uncertainties were propagated using ~~calculated by~~ Gaussian error propagation, and the resulting propagated standard deviations represent

the standard errors of the parameter estimates. ~~and are reported as standard deviation.~~ Comparisons of measured $\epsilon_{\text{Rubisco}}$ values were performed using two-tailed t-tests, with differences considered statistically significant at $p < 0.05$."

rev 2/6 *Figure 2: From the figure and caption alone it is not clear that the dark and light green circles represent the two different Rubisco concentrations. This is only mentioned in line 291-292 in-text.*

We thank the reviewer for this suggestion. We have clarified the figure caption by adding the following sentence at the end of the caption for Figure 2: "...Green and dark-green circles in panel (a) represent assays performed at two different Rubisco concentrations."

rev 2/7 *Table 1: From the table + caption alone it is not clear why the mean ϵRubisco value of S. oleracea is only based on replicates 2 and 3. Only in lines 296 – 300 (in-text) this is clarified, as this paragraph explains which Rubisco concentrations (70 – 80 µg/ml) result in optimal experimental performance. I would recommend adding information to the table caption to clarify this.*

We thank the reviewer for this comment. To clarify the table, we have added the following sentence to the end of the table 1 caption: "...Mean $\epsilon_{\text{Rubisco}}$ value for fully purified *S. oleracea* is calculated from Replicates 2 and 3 only; Replicate 1 yielded insufficient data for a reliable estimate."

rev 2/8 *Line 313: Please also show statistics when stating 'the difference is not statistically significant' (e.g. t-test). This also goes for line 428.*

We agree with the reviewer that statistical support should be explicitly reported when stating that differences are not statistically significant. Accordingly, we have added two-tailed t-tests to all comparisons of measured $\epsilon_{\text{Rubisco}}$ values, including those referred to in Lines 313 and 428. The corresponding statistical results are now reported in the revised manuscript.

rev 2/9 *Considering 70% of proteins is Rubisco for the partially purified extracts, the Rubisco concentration for replicate 2 is likely around 97 µg/ml. Considering this, the vmax value is substantially lower for the partially purified Rubisco as compared to the fully purified Rubisco. What could be the reason for this? Does this mean the Rubisco in the partially purified extract is actually less catalytically active than the Rubisco from the fully purified extract? This would contradict what you state in line 261: "... approximately 63% of the Rubisco in the fully-purified extract and nearly 100% in the semi-purified extract was catalytically active". Do the impurities inhibit the activity of Rubisco? I would recommend clarifying this.*

We thank the reviewer for carefully pointing out this inconsistency. Total protein content, Rubisco concentration, and catalytic activity were assessed using three independent approaches: Bradford assay for total protein concentration, SDS–PAGE gel for Rubisco abundance, and ^{14}CABP-binding assays for the fraction of catalytically active Rubisco.

For the fully purified Rubisco preparation, all three measurements (total protein, Rubisco content, and ^{14}CABP-binding activity) were performed on the same extract that was subsequently used in the isotope fractionation experiments. In contrast, for the semi-purified Rubisco preparation, total protein concentration and Rubisco content were determined on the extract used for the fractionation experiments, whereas the CABP-binding assay was performed on a different semi-purified preparation obtained from an independent extraction. As a result, the ^{14}CABP-based estimate of catalytic activity for the semi-purified extract is not directly linked to the exact preparation used for the kinetic measurements.

We therefore cannot conclusively determine whether the lower apparent v_{max} of the semi-purified Rubisco reflects a reduced fraction of catalytically active enzyme, inhibitory effects of co-purifying proteins, or variability introduced by comparing different preparations. To avoid overinterpretation and speculation, we have removed the statement that "nearly 100 %" of Rubisco in the semi-purified extract was catalytically active. The revised manuscript now only reports activity estimates where measurements were performed on the same extract used for isotope fractionation (see below).

Importantly, this revision does not affect the main conclusions of the study, as the determination of $\epsilon_{Rubisco}$ is independent of v_{max} values.

L260-262: "...Based on the ^{14}C–CABP binding assay, approximately 63% of the Rubisco in the fully-purified extract ~~and nearly 100% in the semi-purified extract~~ was catalytically active. For the semi-purified extract, the ^{14}C–CABP assay indicated a high proportion of active Rubisco, but this estimate was not used quantitatively because the isotope fractionation experiment was performed on a separate extract.."

rev 2/10 *Line 394 - 397: You implemented different values for the inhibitory constant (KI) for S. oleracea, Synechococcus, and G. oceanica, considering S. oleracea and Synechococcus are associated with Form IB, and Gephyrocapsa is associated ID. This is not entirely clear from this sentence. From first reading this sentence it seems the KI value for all species is derived from Rubisco Form ID of Galdieria sulphuraria, although this is only the case for G. oceanica. I would recommend clarifying this.*

We agree with the reviewer that the original wording was slightly ambiguous. We have revised the sentence to explicitly clarify that the inhibitory constant derived from Form ID Rubisco of *Galdieria sulphuraria* was applied only to *G. oceanica*. The revised sentence now reads as follows:

L395-L397: "...For *G. oceanica*, the rate of inhibitory by-product accumulation was comparable to that in *S. oleracea* (see Table 1); however, unlike the Form IB Rubisco of *S. oleracea* and *Synechococcus*, the inhibitory constant used for *G. oceanica* was taken from a Form ID Rubisco — specifically that of *Galdieria sulphuraria* — and ~~applied in our model — derived from Rubisco Form ID of *Galdieria sulphuraria*~~ — is nearly 20 times higher..."

Technical comment

rev 2/11 *Line 84: S. oleracea is not previously mentioned in the introduction using full species name. I would recommend writing it in full species name here, so Spinacia oleracea.*

We agree with the reviewer and have revised the text accordingly.