

Research review

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The diversity and coevolution of Rubisco and CO₂ concentrating mechanisms in marine macrophytes

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Summary

The kinetic properties of Rubisco, the most important carbon-fixing enzyme, have been assessed in a small fraction of the estimated existing biodiversity of photosynthetic organisms. Until recently, one of the most significant gaps of knowledge in Rubisco kinetics was marine macrophytes, an ecologically relevant group including brown (Ochrophyta), red (Rhodophyta) and green (Chlorophyta) macroalgae and seagrasses (Streptophyta). These organisms express various Rubisco types and predominantly possess CO2-concentrating mechanisms (CCMs), which facilitate the use of bicarbonate for photosynthesis. Since bicarbonate is the most abundant form of dissolved inorganic carbon in seawater, CCMs allow marine macrophytes to overcome the slow gas diffusion and low CO2 availability in this environment. The present review aims to compile and integrate recent findings on the biochemical diversity of Rubisco and CCMs in the main groups of marine macrophytes. The Rubisco kinetic data provided demonstrate a more relaxed relationship among catalytic parameters than previously reported, uncovering a variability in Rubisco catalysis that has been hidden by a bias in the literature towards terrestrial vascular plants. The compiled data indicate the existence of convergent evolution between Rubisco and biophysical CCMs across the polyphyletic groups of marine macrophytes and suggest a potential role for oxygen in shaping such relationship.

Introduction

Rubisco (D-ribulose 1,5-bisphosphate carboxylase/oxygenase) is the fundamental link between inorganic and organic matter on Earth. It controls the global carbon cycle by catalysing the first step in the Calvin–Benson–Bassham cycle, fixing CO₂ into ribulose 1,5-bisphosphate (RuBP) and producing two molecules of 3phosphoglycerate (3-PGA). Despite this pivotal role, Rubisco is unable to fully discriminate between CO₂ and its competing substrate, O₂. When Rubisco oxygenates RuBP, it produces 2phosphoglycolate, a toxic product that must be recycled back to 3-PGA via photorespiration, which is considered a wasteful process for being energy-consuming and for requiring the liberation of previously fixed CO₂ (Ort *et al.*, 2015). Rubisco catalysis is further encumbered by its low affinity for CO₂ (K_c) and low carboxylation rate (k_{cat}^{c}), which limit the enzyme's carboxylation efficiency. Due to these catalytic inefficiencies, phototrophic organisms have evolved to invest large amounts of N and energy in the synthesis of Rubisco, allowing them to sustain effective photosynthetic rates. This places Rubisco as one of the most abundant proteins in the world (Bar-On & Milo, 2019).

Rubisco exhibits diverse oligomeric arrangements of its large subunit dimer, giving rise to forms I, II, II/III and III, collectively referred to as *bona fide* Rubiscos. Form I Rubisco is the most widespread and complex in nature and is composed of eight large and eight small subunits, requiring a subclassification into four distinct types (IA, IB, IC and ID; Tabita *et al.*, 2008). Forms IA and IC are the most ancestral Rubisco I forms, whereas forms IB and ID are the most commonly expressed by eukaryotic organisms. Form ID is present in the nongreen algal lineages (rhodophytes, cryptophytes,

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ochrophytes and haptophytes) and form IB in green algal lineages (glaucophytes, euglenozoans and chlorophytes) and streptophytes. The diversity in Rubisco forms suggests an adaptive potential of the enzyme's structure and function, which may have allowed photosynthetic organisms to thrive in a variety of environments.

Despite recent efforts in characterizing the kinetic diversity of Rubisco (Galmés *et al.*, 2014b; Hermida-Carrera *et al.*, 2016; Young *et al.*, 2016; Heureux *et al.*, 2017; Goudet *et al.*, 2020; Aguiló-Nicolau *et al.*, 2023), a larger scale exploration is still required to determine the precise range of variation across different phylogenetic groups. A detailed analysis of existing literature on Rubisco kinetics reveals a marked bias towards Streptophyta, which comprises over 70% of the published data so far (Iñiguez *et al.*, 2020). Consequently, delving further into Rubisco's kinetic properties in nature could reveal undiscovered enzymatic attributes and expose hidden diversity in its catalytic capabilities. Considering that CO₂ availability has emerged as a key factor driving Rubisco evolution (Iñiguez *et al.*, 2020; Cummins, 2021), exploring the enzyme's adaptive evolution in CO₂-limited environments may provide valuable insights into the enzyme's functional diversity.

In seawater, CO₂ diffusion is c. 10 000 times slower than in air (Zeebe, 2011), provoking a severe limitation in the supply of CO₂ to Rubisco. This is worsened by the fact that O2 diffusion in water is also much slower than in air, and therefore, the release of O2 out of the photosynthetic cells is restricted and photorespiration is promoted (Mass et al., 2010). In addition, CO₂ represents < 1% of dissolved inorganic carbon (DIC) in air-equilibrated seawater (Maberly & Gontero, 2017), and its concentration is below the CO2 Michaelis-Menten semi-saturation constant reported for most Rubiscos (Iñiguez et al., 2020). These conditions exacerbate Rubisco catalytic inefficiencies, and hence, it is not surprising that most marine autotrophs have evolved CO₂-concentrating mechanisms (CCMs) allowing them to sustain effective carbon assimilation rates in seawater (Giordano et al., 2005). Therefore, investigating the coevolution of Rubisco and CCMs in marine organisms constitutes a critical research priority to expand the boundaries of Rubisco kinetic adaptability.

Marine macrophytes, which play a crucial ecological role as primary producers in coastal ecosystems world-wide (Duarte et al., 2018), provide an excellent opportunity for such research. These polyphyletic organisms include species of brown (class Phaeophyceae within the phylum Ochrophyta), red (Rhodophyta) and green (Chlorophyta) macroalgae and seagrasses (Streptophyta). Therefore, marine macrophytes represent different key adaptive steps within the oxygenic photosynthetic evolution, from macroalgae groups that have never colonized the terrestrial environment, to seagrasses, the only angiosperm group that successfully colonized seawater. Furthermore, most marine macrophytes have developed CCMs involving biophysical components that actively transport DIC across cellular membranes and/or acidify the extracellular medium, increasing CO₂ concentration near the plasma membrane (Raven & Beardall, 2016). Thus, marine macrophytes serve as a promising group for investigating the coevolution between CCMs and Rubisco.

The operation of biochemical CCMs (C_4 and CAM metabolisms) has not been observed in the vast majority of characterized marine macrophyte species, which are considered C_3 rather than C_4

or CAM species (Koch et al., 2013). Nevertheless, evidence has been found of phosphoenolpyruvate carboxylase (PEPc) or phosphoenolpyruvate carboxykinase (PEPck) activity involved in carbon assimilation in few seagrasses and marine algae (Beer et al., 1980; Reiskind & Bowes, 1991), although Kranz anatomy has not been observed in any marine macrophyte. The biophysical CCMs of marine macrophytes can be facultative, being regulated by several environmental conditions (Giordano et al., 2005). In this sense, the identification and characterization of their CCMs are methodologically challenging and require the use of indirect physiological measurements, especially those that assess the capacity of photosynthetic HCO3⁻ usage (Björk et al., 1997; Hellblom et al., 2001; Beer et al., 2002; Cavalli et al., 2012; Borum et al., 2016; Stepien et al., 2016). Although less information is available for aquatic CCMs compared with those found in terrestrial plants, the coevolutionary trends between Rubisco and CCMs in marine macrophytes have recently gained attention (Iñiguez et al., 2019; Capó-Bauçà et al., 2022a,b, 2023), filling one of the most important gaps of knowledge in Rubisco kinetics in the literature (Iñiguez et al., 2020).

In this review, we delve into the intricate relationship between Rubisco and the biophysical CCMs found in marine macrophytes, placing it within the broader narrative of the evolution of oxygenic photosynthesis. Our work highlights an aspect of Rubisco's evolution that has been overshadowed by a biased literature, which predominantly focuses on terrestrial vascular plants. This approach has often overlooked significant insights that can be gained from the study of marine environments.

Ubiquity of CCM across marine macrophyte groups

The presence of CCMs in marine macrophytes is typically assessed using two indirect techniques: carbon isotope discrimination of organic matter (δ^{13} C) and pH drift assays. The δ^{13} C in seawater is c. -10% for CO₂ and c. 0% for HCO₃⁻, assuming a seawater δ^{13} C for total DIC of 0‰ at 10°C under air-equilibrated condition and an equilibrium isotopic fractionation between dissolved CO₂ and dissolved HCO3⁻ of -10.72 (Mook et al., 1974; Maberly et al., 1992). Consequently, low ¹³C discriminated values in marine macrophyte tissues are often interpreted as a major use of HCO₃⁻ for photosynthesis, indicating the presence of a CCM. By contrast, organisms relying only on diffusive CO2 supply to Rubisco exhibit organic matter significantly depleted in ¹³C due to the discrimination of Rubisco enzyme during CO2 fixation (Hepburn et al., 2011). Therefore, δ^{13} C values lower than -30% are considered a threshold for excluding CCM presence (Maberly et al., 1992), based on the intrinsic ¹³C discrimination value of Rubisco from vascular plants (O'Leary, 1984). Nevertheless, several issues arise using this criterion, as $\delta^{13}C$ in marine macrophytes can be influenced by various factors such as sediment interactions, growth rate, a different intrinsic ¹³C discrimination of Rubisco or CCMs leakage (Raven & Beardall, 2014). For instance, species solely using HCO3⁻ could range from 0.08‰ (assuming HCO₃⁻ entering the cell without discrimination between ¹³C and $^{12}\mathrm{C}$ and no leakage) to -29% if there is substantial leakage of pumped HCO₃⁻ (Maberly et al., 1992).

Alternatively, pH drift assay measures DIC depletion in the medium by tracking the change in seawater pH during incubation within a closed system (Maberly, 1990). When pH exceeds 9, the dissolved CO₂ fraction accounts for nearly 0% of DIC in seawater (< 1 μ mol kg⁻¹ seawater), and thus, any further DIC depletion (i.e. pH increase) indicates the capacity to utilize HCO₃⁻ for photosynthesis. While both metrics indirectly assess CCM presence, the pH drift assay provides an immediate measure of the currently operating CCM, whereas δ^{13} C reflects a time-integrated measurement of CCM activity throughout the organism's lifespan (Stepien *et al.*, 2016).

In the present review, a literature survey was conducted to compile data about δ^{13} C and pH drift assays for macroalgae (Chlorophyta, Rhodophyta and Ochrophyta) and seagrasses, to assess the ubiquity of CCM across marine macrophyte groups (Supporting Information Table S1). Species were also categorized based on their habitat depth when it was reported, considering deep species those that inhabit depths > 10 m and shallow species those that inhabit depths < 10 m. We note that, despite this classification, certain species can thrive across a wide range of depths. Furthermore, the studies analysed employed diverse ecological classification systems to describe species habitats. As a result, certain species had to be excluded from our depth-based classification due to inconsistencies in the literature. For overreported species, the mean of their δ^{13} C values and pH drift compensation points were used in the analysis. A total of 673 species for δ^{13} C and 216 species for pH drift assays were included. δ^{13} C values lower than -30%and pH drift compensation points lower than 9 were established as a cut-off for excluding CCMs.

The results suggest that biophysical CCMs might be commonly expressed by seagrasses and marine macroalgae from the phyla Ochrophyta and Chlorophyta (Fig. 1). Nevertheless, > 30% of δ^{13} C values and > 41% of pH drift compensation points compiled for rhodophyte species indicate that they rely only on diffusive CO_2 supply to Rubisco, and hence, there is a high proportion of non-CCM species in this group (Fig. 1). This result aligns with previous studies, which proposed that certain subtidal and shaded intertidal red algae are associated with an only diffusive CO₂ supply to Rubisco (Maberly, 1990; Raven et al., 2002; Mercado et al., 2009; Cornwall et al., 2015; Diaz-Pulido et al., 2016). When examining each phylogenetic group, a general trend of more depleted ¹³C composition and low pH compensation points was observed in deep marine macrophytes species (Fig. S1), consistent with previous studies (Cornwall et al., 2015; Kübler & Dudgeon, 2015; Stepien et al., 2016). However, it should be noted that this trend may not be universally applicable, as variations can occur depending on both the species and their specific environmental conditions (Marconi et al., 2011; James et al., 2022). Overall, the majority of data reported support the notion that light limitation at greater depths favours a diffusive CO₂ supply to Rubisco, as opposed to the more energetically demanding carbon uptake using CCMs (Raven et al., 2005; Hepburn et al., 2011).

Interpreting the δ^{13} C values and pH compensation points in marine macrophytes can be challenging. For instance, some slowgrowing species might not deplete DIC at a measurable rate during pH drift assays. Additionally, nutrient uptake and sediment interactions can influence δ^{13} C, as observed in certain chlorophyte species of the genus *Caulerpa* (Raven *et al.*, 2002). Despite these challenges, in over 85% of the cases where both δ^{13} C values and pH compensation points have been reported for the same species, both methods concur in their diagnosis (Table S2). Overall, one consistent observation from the indirect proxies compiled is the frequent occurrence of CCMs in marine macrophytes.

Different types of CCMs expressed by marine macrophytes

One of the most common biophysical CCM in aquatic macrophytes is the expression of extracellular carbonic anhydrases (CAs) coupled with H^+ extrusion pumps. This mechanism creates localized acidic zones in the periplasmatic space where CAs facilitate the conversion of HCO_3^- to CO_2 . In these zones, the decrease in the pH can shift the equilibrium of DIC towards CO_2 up to 100-fold relative to air-equilibrium conditions, as observed by Walker *et al.* (1980) in freshwater green algae of the Characeae family. The elevated CO_2 concentration in the periplasmatic space can then diffuse through cellular membranes, and ultimately, provide a higher CO_2 concentration around Rubisco active sites compared with the bulk medium (Fig. 2).

This mechanism can be identified through the inhibition of biophysical CCM components while measuring photosynthetic rates, by using CA inhibitors such as acetazolamide (AZ) or pH buffers that dissipate H⁺ gradients (Beer et al., 2002; Larkum et al., 2017). Over the last decades, evidence has been collected supporting the operation of this mechanism in various macroalgal (Koch et al., 2013; Capó-Bauçà et al., 2023) and seagrass species (Hellblom et al., 2001; Beer et al., 2002; Uku et al., 2005; Borum et al., 2016; Capó-Bauçà et al., 2022a,b), indicating that this type of CCM is commonly expressed by marine macrophytes. This mechanism has been proposed to be particularly specialized in seagrasses, possibly due to transfer cell-like structures in their epidermis (Larkum et al., 2017). These structures may enhance the effectiveness of their CCMs, allowing seagrasses to concentrate CO₂ around Rubisco to a higher concentration than the ambient during steady-state photosynthesis. This could explain why seagrasses are more effective in concentrating CO₂ relative to their brackish water and freshwater counterparts (Capó-Bauçà et al., 2022a).

Another type of biophysical CCM operating in marine macrophytes is the active transport of DIC across cellular membranes. This method consists of an active HCO_3^- acquisition by protein transporters followed by a conversion into CO_2 in the intracellular medium. If this conversion is done within the chloroplast stroma, it could potentially increase CCM effectiveness, since it may reduce CO_2 leakage out of the cell for being closer to Rubisco active sites (Fig. 2). One way to detect these CCMs is using stilbene inhibitors of anion exchange transporters, like 4,4'-di-isothiocyanatostilbene-2,2'-disulfonate (DIDS) or 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS), a method first used by Drechsler & Beer (1991) in *Ulva latuca*. Using this method, several macroalgae species have been identified as DIDS-sensitive (Drechsler *et al.*, 1993; Raven & Hurd, 2012;





Fig. 1 Estimated percentage of marine macrophyte species exhibiting CO₂ concentrating mechanisms (CCMs), inferred from compiled δ^{13} C and pH drift assay data in Supporting Information Table S1. δ^{13} C values lower than -30% and pH drift compensation points lower than 9 were established as a cut-off for excluding CCMs.



Fig. 2 Diagram of the different components of CO_2 concentrating mechanisms operating in marine macrophytes. CA_{ext} , extracellular carbonic anhydrases; CA_{int} , intracellular carbonic anhydrases; T, ATPase-mediated HCO_3^- transporters; P, H⁺ extrusion pumps. Diagram based on previously published work by Giordano *et al.* (2005).

Fernández *et al.*, 2014; Capó-Bauçà *et al.*, 2023). Contrarily, the impact of anion exchange inhibitors on seagrass CCMs has been minimal (Rubio *et al.*, 2017; Capó-Bauçà *et al.*, 2022a). Nevertheless, active HCO_3^- acquisition mediated by unsensitive DIDS transporters has been proposed for several seagrass species (Hellblom *et al.*, 2001; Beer *et al.*, 2002). Rubio *et al.* (2017) demonstrated the operation of fusicoccin-sensitive H⁺-ATPase as the main plasma membrane energization system in *Posidonia oceanica*, generating an electrochemical gradient that drives the direct active uptake of HCO_3^- .

While the cellular components of CCMs are better understood in some model microalgae compared with marine macrophytes (Burlacot & Peltier, 2023), both groups exhibit a diverse array of biophysical CCMs facilitating DIC acquisition. This diversity may suggest that marine macrophyte CCMs might vary in their efficiency to concentrate CO_2 near Rubisco active sites.

CCM effectiveness across marine macrophyte groups

Irrespective of the type of CCM operating, a key issue in marine macrophytes is whether their photosynthetic rate is saturated by seawater CO_2 or DIC concentrations under present-day conditions. This can be assessed using the *in vivo* photosynthetic Michaelis–Menten constants for CO_2 or for DIC (K_m and K_m _{DIC},



Fig. 3 Boxplots of the photosynthetic semi-saturation constants for dissolved CO_2 (a) and inorganic carbon (DIC, b) of the data compiled in Supporting Information Table S3. Black lines inside the boxes indicate the data median, the bounds of the boxes delimit the 25^{th} – 75^{th} percentiles of the data, the whiskers represent the $1.5 \times$ interquartile range limits and the dots represent the data beyond the end of the whiskers (outliers). Different small letters show significant differences among species (P < 0.05, Kruskal–Wallis test followed by Bonferroni correction). *Pa, Posidonia angustifolia*.

respectively), which can be used as a proxy for the ability to use inorganic carbon for photosynthesis by marine macrophytes. Nevertheless, it is important to consider some underlying complexities of this proxy. For example, K_m values are calculated from the proportion of CO₂ within DIC at a given pH and temperature, but the value is probably largely determined by HCO₃⁻ uptake in species where this form is used. Thus, while these constants are valuable for comparative assessments among species, one must exercise caution when interpreting their direct physiological implications in natural settings.

In the present review, a compilation of $K_{\rm m}$ and $K_{\rm m DIC}$ published in the literature for macroalgae (Chlorophyta, Rhodophyta and Ochrophyta) and seagrasses was also undertaken (Table S3). For overreported species, the mean of their $K_{\rm m}$ and $K_{\rm m DIC}$ values was used in the analysis. In total, 42 species for $K_{\rm m}$ and 74 species for $K_{\rm m}$ DIC were included. Based on the data compiled, all marine macrophytes groups showed a similar affinity for DIC, whereas chlorophyte macroalgae presented the highest affinity for CO₂ (Fig. 3). However, significant variability was observed across species within the same phylogenetic group, indicating that the observed effects were likely due to species-specific traits rather than broad phylogenetic patterns (Fig. 3; Table S3). *Posidonia angustifolia*, a seagrass species found at a depth of 20 m, exhibited the highest K_m and K_m _{DIC} values compared with other species (Fig. 3). The low irradiance conditions in the habitat of *P. angustifolia* may limit the energy available for its CO₂ concentrating mechanisms, which could explain its relatively low CCM activity compared with other species within the same phylogenetic group (Capó-Bauçà *et al.*, 2022b).

We must highlight that some of the $K_{\rm m}$ and $K_{\rm m}$ DIC compiled here were determined using buffers to maintain constant pH values during the measurements (see Table S3). The acidification of localized zones of the periplasmic space is a buffer-sensitive CCM that usually operates in marine macrophytes, so the use of pH buffers overestimates $K_{\rm m}$ and $K_{\rm m}$ DIC values in those organisms and hence underestimates their affinity for DIC and CO₂ (Price & Badger, 1985; Hellblom *et al.*, 2001). The analysis of the data conducted without using pH buffers did not reveal significant differences in the $K_{\rm m}$ and $K_{\rm m}$ DIC values between seagrasses and macroalgae ($K_{\rm m}$ *P*-value = 0.183 and $K_{\rm m}$ DIC *P*-value = 0.405, obtained from Welch two-sample *t*-test), rejecting an underlying hypothesis in the literature of a less effective CCMs in seagrasses than in macroalgae.

Beer (1989, 1994) and subsequent studies (see Koch et al., 2013) suggested that seagrasses must be DIC limited under current atmospheric conditions, as they increased net photosynthesis, reproduction, belowground biomass and the production of nonstructural carbohydrates under elevated CO₂ levels. However, the positive acclimatory response to high CO₂ is not itself evidence of a less effective CCM in seagrasses. This response might be related to reduced respiration rates and energy savings due to CCM deactivation promoting higher growth (Gordillo et al., 2001; Iñiguez et al., 2016) or reproduction (Jiang et al., 2010). A wealth of evidence is compiled in this study indicating a remarkable specialization of CCMs in both seagrasses and marine macroalgae, with most exhibiting nearly saturated photosynthetic rates under present-day seawater DIC concentrations (Table S3). Therefore, investigating this complex array of CCMs can yield valuable insights into the adaptive mechanisms of marine macrophytes and their contribution to the global carbon cycle.

Marine macrophytes diverge from canonical Rubisco trade-offs

Over the last decades, the idea that Rubisco exhibits inherent compromises in its enzymatic attributes has been established, delimiting a boundary for its kinetic optimization (Tcherkez *et al.*, 2006; Savir *et al.*, 2010). These inherent compromises, known as catalytic trade-offs, primarily involve the inverse relationships between Rubisco affinity for CO₂ (1/ K_c) and k_{cat}^{c} , and between Rubisco specificity factor (S_{clo}) and k_{cat}^{c} . For instance, the increase in k_{cat}^{c} observed during the evolution of C₄ and CAM plants comes at the expense of reduced Rubisco affinity for CO₂, as a result of Rubisco adaptation to the high intra chloroplastic CO₂



Fig. 4 Trade-offs between the Rubisco kinetic parameters at 25°C for the eukaryotic organism compiled by Goudet *et al.* (2020), Iñiguez *et al.* (2020) and Capó-Bauçà *et al.* (2022a,b, 2023). (a) Trade-off between Rubisco maximum carboxylation rate (k_{cat}^{c}) and *in vitro* Rubisco Michaelis–Menten constant for CO₂ (K_c); (b) Trade-off between Rubisco specificity factor ($S_{c/o}$) and K_c ; (c) Trade-off between $S_{c/o}$ and k_{cat}^{c} ; (d) trade-off between the *in vitro* Rubisco Michaelis–Menten constant for O₂ (K_o); (b) Trade-off between the *in vitro* Rubisco are highlighted in green. Different symbols were used to differentiate phylogenetic groups. Statistically significant correlations either for non-marine plants or for marine macrophytes are shown (*P*-values were obtained from a two-sided test for association between paired samples based on Spearman's rank correlation ρ , *, *P* < 0.01; ***, *P* < 0.001).

concentration provided by the biochemical CCMs (Whitney *et al.*, 2011; Kapralov *et al.*, 2012; Sharwood *et al.*, 2016; Hermida-Carrera *et al.*, 2020). Conversely, this kinetic trade-off is also responsible for the opposite trend seen in Rubisco expressed by C_3 drought-resistant plants, where a low leaf diffusive conductance to CO_2 imposes a strong restriction (Galmés *et al.*, 2014a). These results suggest that Rubisco kinetics can be fine-tuned to intracellular CO_2 concentrations, even within short evolutionary timescales throughout the plant phylogeny.

Attending to the fact that these catalytic trade-offs are intrinsically linked to Rubisco reaction (Tcherkez & Farquhar, 2021), one will expect similar Rubisco adaptative patterns in all photosynthetic organisms expressing CCMs. However, in the last few years, novel Rubisco kinetics data has been reported for bacteria (Davidi *et al.*, 2020), unicellular algae (Young *et al.*, 2016; Goudet *et al.*, 2020) and marine macrophytes (Iñiguez *et al.*, 2019; Capó-Bauçà *et al.*, 2022a,b, 2023), emerging a distinct pattern of

New Phytologist (2024) 241: 2353–2365 www.newphytologist.com kinetic trade-offs compared with terrestrial vascular plants (Flamholz et al., 2019; Iñiguez et al., 2020). This expansion of knowledge revealed divergences in the catalytic trade-offs across Rubisco forms and among phylogenetically distinct groups, driven by the strength and direction of selective pressures encountered during their evolution (Griffiths et al., 2017; Young & Hopkinson, 2017; Cummins, 2021). Specifically, marine macrophytes exhibit a lower slope in the correlation between k_{cat}^{c} and K_{c} , and no significant correlation was found between $S_{c/o}$ and K_c , and between $S_{c/o}$ and k_{cat}^{c} (Fig. 4a–c). Additionally, marine macrophytes displayed a lack of correlation between CO2 and O2 affinities (Fig. 4d), suggesting that not only intracellular CO_2 but also O2 concentration could be another important driver for their Rubisco kinetic evolution. This observation aligns with the reported higher internal oxygen concentrations in marine macrophytes (Kim et al., 2018), indicating a distinctive evolutionary adaptation of their Rubisco kinetics. This distinctive catalytic pattern mirrors the trends observed in unicellular algae, which includes organisms belonging to the phyla Haptophyta, Charophyta and unicellular algae from the phyla Chlorophyta, Rhodophyta and Ochrophyta, some of them presenting even a wider deviation in the Rubisco kinetic trade-offs than marine macrophytes (Fig. 4).

These findings support the idea of a greater plasticity in Rubisco kinetics than previously thought, emphasizing a notable bias in Rubisco literature towards terrestrial vascular plants. Bouvier et al. (2021) went a step further, suggesting that there might be a potential overestimation of the Rubisco catalytic trade-off in previous plant-based datasets that did not consider the phylogenetic relationships of the species. Importantly, the study of Bouvier et al. (2021) also demonstrated that weaker trade-offs in Rubisco are present not only in land plants, but also across a variety of photosynthetic organisms when considering the phylogenetic relationships of the species. Although the phylogenetic dependence of Rubisco trade-offs continues to be a topic of debate (Tcherkez & Farquhar, 2021; Bouvier & Kelly, 2023), our findings underline that the established paradigm of Rubisco kinetic trade-offs, grounded primarily in studies on terrestrial vascular plants, is not universally applicable, even for angiosperms like seagrasses.

Coadaptation of Rubisco kinetics and CCMs in marine macrophytes

During evolution, Rubisco has been selected towards increasing its carboxylation efficiency and specificity for CO2 (Iñiguez et al., 2020; Bouvier et al., 2023). Form I Rubisco increased its affinity and specificity for CO₂ compared with the ancestral forms II, II/III and III, in response to the decrease in CO2 atmospheric concentration and increase in O2 atmospheric concentration during oxygenic photosynthetic evolution (Shih et al., 2016; Schulz et al., 2022). However, some exceptions to this general trend have been observed. Lin et al. (2022) identified several ancestral Rubiscos in the Solanaceae family with higher k_{cat}^{c} and carboxylation efficiency than their modern counterparts. The most notable exception, proposed by Meyer & Griffiths (2013), suggests that the high CO2 conditions near Rubisco active sites provided by CCMs have reduced the selective pressure for higher Rubisco carboxylation efficiency, proportionally to the prolonged time of coexistence between CCMs and Rubisco.

The coexistence of Rubisco with CCMs possibly began during the late Archaean or early Proterozoic in mats and stromatolites of cyanobacteria, *c*. 2500 million years ago (Ma) (Riding, 2006). A subsequent development of CCMs is proposed to have occurred in eukaryotic algae during the mid-Phanerozoic period, 400–300 Ma, a time characterized by the lowest atmospheric $CO_2: O_2$ ratio (Raven *et al.*, 2008). Furthermore, more recent development of CCMs has been reported in terrestrial vascular plants, suggesting that CAM photosynthesis likely evolved in plants living in seasonal pools in the Mesozoic era, *c*. 200 Ma (Keeley & Rundel, 2003). Lastly, C₄ plants represent an even more recent innovation, with this group not radiating until the last drop in atmospheric CO_2 concentration *c*. 30 Ma (Sage *et al.*, 2018). These developments illustrate the polyphyletic origin of CCMs and the varied timescales



Fig. 5 Upper panel, evolutionary scheme of the oxygenic photosynthesis in the red and green lineage. Lower panel, Rubisco carboxylation efficiency $(k_{cat}{}^{c}/K_{c})$ in different groups of photosynthetic organisms. In this panel, values are means \pm SE and different lowercase letters show significant differences among groups (*P* < 0.05; Kruskal–Wallis test followed by Bonferroni correction).

of coexistence of CCMs with Rubisco across different groups of photosynthetic organisms.

The Rubisco kinetic data compiled in this study corroborate the hypothesis of Meyer & Griffiths (2013), with a steady increase in Rubisco carboxylation efficiency across the green lineage, from cyanobacteria to vascular plants and according to the different times of coexistence between CCMs and Rubisco (Fig. 5). Remarkably, a gradual decrease in Rubisco carboxylation efficiency was observed during the hypothetical angiosperm seawater colonization, a steady process from freshwater to brackish water and, finally, to seawater environments (Les *et al.*, 1997). Notable, this trend suggests a divergence from the established patterns observed across the evolution of green lineage, highlighting a distinct adaptive mechanism of seagrasses. Indeed, the average Rubisco carboxylation efficiency in seagrasses is as low as the values reported for eukaryotic nongreen algae (Fig. 5).

Seagrasses diverged from their ancestors c. 100 Ma during the colonization of the sea (Wissler *et al.*, 2011), and possibly radiated c. 70–60 Ma (Chen *et al.*, 2022). While seagrasses might not have

2360 Review

 $K_{\rm c}^{\rm air}/K_{\rm m}$ (s $\mu {
m M}^{-1}$

7.5

2.5

0.0

50

Research review





been the first plants to develop CCMs, as other aquatic angiosperms possibly adopted simple versions of CAM mechanisms earlier (Keeley & Rundel, 2003), the considerable period of coexistence between Rubisco and CCMs, coupled with the low CO_2 availability in seawater environments, may have influenced Rubisco seagrass evolution. This fact could have led seagrasses to either not develop or to reverse most of the enhancement in Rubisco carboxylation efficiency gained during terrestrial plant evolution and to converge with the kinetic properties of CCM-expressing nongreen algae species (Capó-Bauçà *et al.*, 2022a).

100

 $k_{\rm cat}^{\rm c}/K_{\rm c}^{\rm air}$ (s $\mu {\rm M}^{-1}$)

 $R^2 = 0.78^{***}$

 ∞

200

150

A lower Rubisco carboxylation efficiency was also found in nongreen algae possessing pyrenoids relative to nongreen algae lacking this structure (Fig. 5). This is likely due to the relationship between the presence of pyrenoid with the operation of effective biophysical CCMs (Badger et al., 1998). This finding indicates that the presence of biophysical CCMs leads to a decrease in the Rubisco carboxylation efficiency in a diverse group of organisms, denoting a convergent evolution across polyphyletic organisms expressing biophysical CCMs. However, the influence of CCMs on Rubisco carboxylation largely depends on their effectiveness in concentrating CO₂ rather than merely their presence (Tortell, 2000). Some species have CCMs that only partially compensate for the slow CO₂ diffusion in aquatic environments, rather than significantly elevating CO2 at the Rubisco active sites over the environmental concentration. Thus, the true impact of CCMs on Rubisco's carboxylation hinges on this crucial distinction.

Since direct measurements of CO₂ concentration around Rubisco are not available, the most accurate way to assess the effectiveness of CCMs in concentrating CO₂ around Rubisco is by using the ratio between the *in vitro* Rubisco semi-saturation constant for CO₂ under air conditions (K_c^{air}) and the *in vivo* photosynthetic semi-saturation constant for CO₂ (K_m , Raven *et al.*, 2017). This ratio has been evaluated in marine macrophytes in recent studies by Capó-Bauçà *et al.* (2022a,b, 2023). A negative relationship between the Rubisco carboxylation efficiency under ambient O₂ concentration (K_c^{air}/k_{cat}^c) and CCMs effectiveness (K_c^{air}/K_m) was observed across aquatic macrophyte species (Fig. 6), demonstrating that biophysical CCMs, and therefore the intra chloroplastic CO_2 level, is one of the most important drivers shaping Rubisco evolution in these organisms.

The prolonged time of coexistence between CCMs and Rubisco, as well as the effectiveness of CCMs, could explain the low Rubisco carboxylation efficiency found in most marine macrophytes. Nevertheless, it could also be related to the O₂ concentration in the proximity of their Rubisco active sites. The low Rubisco carboxylation efficiency (k_{cat}^{c}/K_{c}) found in marine macrophytes was closely associated with a proportionally low Rubisco oxygenation efficiency (k_{cat}^{o}/K_{o}) , a correlative pattern found across many photosynthetic organisms (Fig. 7). This may indicate that marine macrophytes evolved towards low k_{cat}^{o}/K_{o} at the expense of an irremediable decrease in k_{cat}^{c}/K_c , which could be compensated by the development of CCMs. Such an evolutionary trend could be a response to the challenges posed by the slow gas diffusion in marine environments. This slow gas diffusion can restrict the liberation of O₂ out of the photosynthetic tissues during photosynthesis (Roberts & Moriarty, 1987; Larkum et al., 2017; Kim et al., 2018), leading to unsustainable rates of photorespiration and compromising the carbon balance in marine macrophytes. In this regard, a low Rubisco carboxylation efficiency in marine macrophytes may be related to the need to reduce the oxygenation reaction of Rubisco.

Interestingly, form ID Rubisco presented a distinct slope than form IB in the correlation between k_{cat}^{c}/K_c and k_{cat}^{o}/K_o (*P*-value of = 5.65 × 10⁻⁶ in ANCOVA analysis; Fig. 7), suggesting that species expressing form ID Rubisco may have evolved enhancing k_{cat}^{c}/K_c over k_{cat}^{o}/K_o more effectively than those expressing form IB. This finding explains the high $S_{c/o}$ values observed in some species expressing form ID Rubisco. The best examples of this assertion are the thermoacidophile red microalga *Galdieria* sulphuraria and the temperate red macrophyte *Griffithsia monilis* (Fig. 7), both expressing form ID Rubisco. The absence of CCMs in *G. monilis* (Raven, 1997; Raven *et al.*, 2002), and the very low DIC availability in the hot and acidic aquatic environments inhabited by *G. sulphuraria* (Curien *et al.*, 2021), likely led to the enhancement of k_{cat}^{c}/K_c over k_{cat}^{o}/K_o in these species. This

Review 2361



Fig. 7 Correlation between Rubisco carboxylation efficiency (k_{cat}^{c}/K_{c}) and Rubisco oxygenation efficiency (k_{cat}^{o}/K_{o}) across the species presented in Fig. 4. Green and pink dotted lines represent the correlations obtained in the ANCOVA analysis for form IB and ID, respectively. Gm, *Griffithsia monilis*; Gs, *Galdieria sulphuraria*.

adaptation suggests that Rubisco carboxylation has been optimized for the specific physiology and environmental factors faced by *G. sulphuraria* and *G. monilis*, resulting in $S_{c/o}$ values ranging among the highest reported to date.

The presence of species like G. sulphuraria and G. monilis, and the distinct evolutionary pathway of macroalgae expressing form ID Rubiscos, relative to those expressing form IB Rubiscos, may indicate a better adaption of form ID Rubiscos to the intracellular CO₂: O₂ ratio found in algal cells during steady-state photosynthesis. This hypothesis is supported by Rickaby & Eason Hubbard (2019), who suggest that the evolution of a high $S_{c/0}$ in organisms expressing form ID Rubisco presumably led to the replacement of the major ocean primary producers, form IBcontaining green algae by form ID-containing haptophytes and heterokonts, at the beginning of the Mesozoic era (c. 250 Ma). Overall, the observed evolutionary patterns in Rubisco forms IB and ID, as well as the variations in carboxylation efficiency among different lineages, underscore the remarkable adaptability of this crucial enzyme and denote the intricate nature of Rubisco's evolutionary history.

Implications of the coadaptation of Rubisco and CCMs in marine macrophytes

Examining Rubisco kinetics and their coadaptation with CCMs in marine macrophytes can provide valuable insights into optimizing Rubisco performance in crops. For instance, Griffithsia monilis exhibited a Rubisco with a higher assimilation capacity (A_{Rub}) in the range of C_c (chloroplastic CO₂ concentration) usually observed in C_3 crops during steady-state photosynthesis than the Rubisco assimilation capacity found in C $_3$ or C $_4$ /CAM plants (Fig. 8). Specifically, few form ID Rubiscos, particularly those from some rhodophyte species, showed catalytic properties with the potential to enhance photosynthesis in crops due to a combination of high $S_{c/o}$ values, high CO₂ affinity and high Rubisco carboxylation efficiency (Whitney et al., 2001). In fact, recent advances in this area have been made by exploring the potential of red ID Rubisco to enhance crop photosynthesis (Oh et al., 2023). Key works by Gunn et al. (2020) and Zhou et al. (2023) have shown promising results using form IC from the proteobacterium Rhodobacter sphaeroides. This form

is phylogenetically related to form ID Rubisco and has fewer folding and assembly requirements, making it a suitable scaffold for introducing engineered red Rubiscos in crops.

Nevertheless, except for *Griffithsia monilis*, all other marine macrophytes analysed presented lower A_{Rub} than those found in C₃ or C₄/CAM plants, in both high and low C_{c} concentrations (Fig. 8a,b, respectively). This implies that the improved carboxylation properties of form ID Rubisco may be confined to low-light environments or to acidic hot springs where CCM activity is either minimal or absent. Marine macrophytes have evolved Rubisco under conditions vastly different from those experienced by terrestrial plants, leading to kinetic profiles not necessarily suitable for enhancing photosynthesis in crops. However, these kinetic profiles can significantly influence the response of marine macrophytes to environmental changes, indicating that the coadaptation of Rubisco with CCMs in these organisms has implications beyond its mere applicability of enhancing crop photosynthesis.

Considering this, our study offers valuable clues into predicting the behaviour of marine macrophytes under the near-future global change scenarios. The ongoing threats of ocean warming and acidification are altering resource ratios and environmental conditions, differently impacting macrophyte species and transforming coastal reef ecosystems (Duarte et al., 2018). On a physiological level, ocean CO2 enrichment is expected to reduce the metabolic cost of inorganic carbon acquisition and fixation in all marine macrophytes, either by potentially downregulating CCMs or by decreasing the Rubisco oxygenase activity in non-CCM species (Hepburn et al., 2011). Nevertheless, under elevated CO₂ conditions, the operation of CCMs should remain advantageous for light-saturated shallower species, but at deeper habitats where energy constrains the functioning of CCMs, non-CCM species will benefit more from a high seawater CO2: O2 ratio than CCMexpressing species. This could lead to an expansion of non-CCM species to brighter and shallower habitats (Kübler & Dudgeon, 2015). In addition, the expansion of non-CCM species could be greater than previously hypothesized due to the high Rubisco carboxylation efficiency of non-CCM species compared with those expressing these mechanisms (Fig. 6).

However, it is the combined effect of CO_2 enrichment and ocean warming that will likely determine the inorganic carbon

2362 Review

New Phytologist



Fig. 8 Rubisco gross assimilation rate (A_{Rub}) at 25°C of the different marine macrophyte groups and *Griffithsia monilis*, along with terrestrial vascular plants. A_{Rub} was calculated using the model of Farquhar *et al.* (1980). To fit the model, we used the mean of each Rubisco kinetic trait under the gas phase measured for each group and assumed an active Rubisco concentration of 1 g m⁻² (or 14.55 μ mol active sites m⁻²) to calculate $V_{c,max}$ (μ mol m⁻² s⁻¹) from k_{cat} ^c (s⁻¹). C_c is the chloroplastic CO₂ concentration, which ranges from 0 to 5000 ppm (a) and from 0 to 300 ppm (b).

acquisition and assimilation mechanisms in marine macrophytes. As temperatures rise, the solubility of gases, including CO₂, decreases in seawater (Maberly & Gontero, 2017). This could partially offset the effects of ocean acidification by reducing the amount of available dissolved CO₂, keeping the ecological fitness of species with CCMs. Furthermore, with rising temperatures, both the K_c and k_{cat}^{c} increase, leading to a higher Rubisco carboxylation rate but a reduced affinity for CO₂, while S_{c/o} decreases. These alterations in Rubisco kinetics suggest that species with efficient CCMs might hold an advantage over non-CCMs species, as CCMs could help mitigate the reduced Rubisco affinity and specificity for CO₂ expected with rising temperatures (Orr et al., 2016). However, it is crucial to consider the thermal dependency of these kinetic parameters, which is species-specific (Galmés et al., 2016). Further research is still required, especially those focused on the effects of temperature on the facultative regulation of CCMs in marine macrophytes, as well as in determining the thermal dependencies of their Rubisco kinetics and the thermal inactivation of their Rubisco activase proteins, topics that still remain largely unexplored in marine macrophytes (Koch et al., 2013; Iñiguez et al., 2020; Capó-Bauçà et al., 2022b). Additionally, the subsequent impacts on the entire life cycle of the species and their long-term acclimatory responses are also almost unknown, necessitating further research to accurately model marine macrophyte responses to global change.

In conclusion, the coadaptation of Rubisco and CCMs in marine macrophytes is not just a mechanism for optimizing enzymatic functions but a complex adaptive response to diverse environmental conditions, with implications extending from ecological balances in marine ecosystems to potential applications in agricultural productivity. Our analysis challenges and expands the previously established paradigm predominantly based on terrestrial vascular plants. The mounting body of evidence up to date (Young *et al.*, 2016; Flamholz *et al.*, 2019; Iñiguez *et al.*, 2021; Davidi *et al.*, 2020; Goudet *et al.*, 2020; Bouvier *et al.*, 2021, 2023;

Capó-Bauçà *et al.*, 2022a,b, 2023) highlights the necessity for a more extensive and diverse Rubisco research. Such continued efforts are crucial for deepening our understanding of this enzyme's adaptation and evolution across different life forms, which remains an important focus for future work in the field.

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Competing interests

None declared.

Author contributions

CI and JG conceived and designed the study. SC-B analysed the data and produced the figures and wrote most of the manuscript with help from all authors.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Percentage of species exhibiting CO_2 concentrating mechanisms (CCMs) for each phylogenetic group of marine macrophytes differentiating those living in deep habitat (>10 m depth) and shallow habitats (<10 m depth).

 $\label{eq:stable} \begin{array}{l} \textbf{Table S1} \ Compilation \ of marine macrophytes \ \delta^{13}C \ values \ and \ pH \\ compensation \ points \ from \ literature. \end{array}$

Table S2 Comparison of the diagnoses (CCM presence or absence) in species reporting both δ^{13} C values and pH compensation points.

Table S3 Compilation of the photosynthetic semi-saturation constants for DIC and CO_2 of marine macrophytes taken from literature.

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