



# Physiological control on carbon isotope fractionation in marine phytoplankton

Karen M. Brandenburg<sup>1</sup>, Björn Rost<sup>2,3</sup>, Dedmer B. Van de Waal<sup>4</sup>, Mirja Hoins<sup>1,2</sup>, Appy Sluijs<sup>1</sup>

<sup>1</sup>Department of Earth Sciences, Faculty of Geosciences, Utrecht University, Princetonlaan 8a, 3584 CB Utrecht, the Netherlands

<sup>2</sup>Department of Marine Biogeoscience, Alfred Wegener Institute (AWI), Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany

<sup>3</sup>Faculty of Biology/Chemistry, University of Bremen, Leobener Strasse, 28359 Bremen, Germany

<sup>4</sup>Department of Aquatic Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Droevendaalsesteeg 10, 6708 PB Wageningen, the Netherlands

Correspondence to: Karen M. Brandenburg (k.m.brandenburg@uu.nl)

**Abstract.** One of the great challenges in biogeochemical research over the past half a century has been to quantify and understand the mechanisms underlying stable carbon isotope fractionation ( $\epsilon_p$ ) in phytoplankton in response to changing  $p\text{CO}_2$ . Partly, this interest is grounded in the use of fossil photosynthetic organism remains as a proxy for past atmospheric  $\text{CO}_2$  concentrations. Phytoplankton organic carbon is depleted in  $^{13}\text{C}$  compared to its source because of kinetic fractionation by the enzyme RubisCO during photosynthetic carbon fixation, as well as through physiological pathways upstream of RubisCO. Moreover, other factors such as nutrient limitation, variations in light regime as well as phytoplankton culturing systems and inorganic carbon manipulation approaches may confound the influence of  $\text{CO}_2$  on  $\epsilon_p$ . Here, based on experimental data compiled from the literature, we assess which underlying physiological processes cause the observed differences in  $\epsilon_p$  for various phytoplankton groups in response to C-demand/C-supply and test potential confounding factors. Culturing approaches and methods of carbonate chemistry manipulation were found to best explain the differences in  $\epsilon_p$  between studies, although daylength was an important predictor for  $\epsilon_p$  in haptophytes. Extrapolating results from culturing experiments to natural environments and for proxy applications therefore requires caution, and it should be carefully considered whether culture methods and experimental conditions are representative of natural environments.

## 1 Introduction

Understanding of past climates, in particular variations in atmospheric  $\text{CO}_2$  concentrations and concomitant temperatures, may help to improve climate models and constrain the global temperature response to projected  $\text{CO}_2$  rise (Rohling et al., 2012; Zhu et al., 2020; Tierney et al., 2020). Reconstructions of past  $\text{CO}_2$  concentrations beyond the reach of ice cores rely on proxy estimates. These are based on biogeochemical relations between  $\text{CO}_2$  concentrations in the atmosphere and the chemical or morphological properties of biogenic carbonates, other minerals, fossil leaves or various types of organic matter that can be found in sediments (Foster et al., 2017; Macdonald, 2020). All these proxies are based on assumptions and exhibit large uncertainties that are ideally constrained iteratively.



One line of proxies uses the CO<sub>2</sub>-dependence of <sup>13</sup>C fractionation during photosynthetic carbon fixation in phytoplankton (O'Leary, 1984; Sharkey and Berry, 1985; Farquhar et al., 1989). Several components found in sediments have been proposed for this, including bulk organic matter (Hayes et al., 1999) and algae-derived molecules, such as porphyrins (Freeman and Hayes, 1992) and phytane (Bice et al., 2006) produced by all photosynthetic organisms. Also, the potential of more specific proxies has been tested, including alkenones originating from coccolithophores (Jasper and Hayes, 1990) and resting cysts produced by dinoflagellates (Hoins et al., 2015; Sluijs et al., 2018). The δ<sup>13</sup>C signal of various algal remains is thought to follow the δ<sup>13</sup>C signal of dissolved inorganic carbon (DIC), in particular the δ<sup>13</sup>C signal of CO<sub>2</sub>, modulated by CO<sub>2</sub>-dependent fractionation during photosynthetic carbon fixation. Therefore, ultimately, the δ<sup>13</sup>C signal in algal fossil remains may be used for estimating atmospheric CO<sub>2</sub> levels through geological time. Accurate use of this proxy relies on the mechanistic understanding of carbon isotope fractionation (ε<sub>p</sub>) in phytoplankton, which is obtained by different culturing approaches and assays targeting relevant physiological pathways.

Fractionation occurs during fixation of CO<sub>2</sub> by ribulose-1,5-bisphosphate carboxylase/ oxygenase (RubisCO) (Raven and Johnston, 1991), and is further dependent on the C-supply to this enzyme as well as the C-demand of the cells (Rau et al., 1996; Bidigare et al., 1997; Hoins et al., 2016). RubisCO discriminates against the <sup>13</sup>C isotope resulting in biomass being <sup>13</sup>C-depleted relative to its source CO<sub>2</sub>. In higher plants, the intrinsic fractionation value of RubisCO (ε<sub>f</sub>) is estimated to be between 26-30‰ (Roeske and O'Leary, 1984; McNevin et al., 2007). However, ε<sub>f</sub> can differ between phytoplankton taxa and species (e.g., Maberly et al., 1992; McNevin et al., 2007), and is indeed an important source of variation in <sup>13</sup>C fractionation among phytoplankton groups. Several catalytically and phylogenetically distinct forms of RubisCO in phytoplankton exist, including Forms IA, IB, ID and II (Whitney et al., 2011; Tabita et al., 2008). Direct *in vitro* measurements of ε<sub>f</sub> yielded values of ~11‰ for the haptophyte *Emiliania huxleyi* (Boller et al., 2011) and ~18.5‰ for the diatom *Skeletonema costatum* (Boller et al., 2015). Much higher fractionation values have been estimated from *in vivo* experiments under nitrate-limited conditions, with values as high as ~25‰ for the diatoms *Phaeodactylum tricornerutum* and *Porosira glacialis*, and for *E. huxleyi* (Popp et al., 1998), and ~27‰ for the dinoflagellate *Alexandrium tamarense* (Wilkes et al., 2017).

These large differences between phytoplankton groups and across treatments point towards physiological processes that can affect fractionation, notably those involved in so-called carbon concentrating mechanisms (CCMs). CCMs have evolved over time as a response to declining atmospheric CO<sub>2</sub> concentrations to ensure effective carboxylation in the vicinity of RubisCO in oxygenated waters (Giordano et al., 2005). Phytoplankton CCMs comprise a variety of physiological adaptations, and include active uptake of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>, the use of carbonic anhydrase (CA) to accelerate the interconversion between CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>, and ways to minimize the CO<sub>2</sub> efflux from the cell (Badger et al., 1998; Reinfelder, 2011; Rokitta et al., 2022). These processes can strongly influence <sup>13</sup>C fractionation patterns of phytoplankton (Sharkey and Berry, 1985). For instance, HCO<sub>3</sub><sup>-</sup> is enriched in <sup>13</sup>C relative to CO<sub>2</sub> (by ~10‰), and a high uptake and assimilation of HCO<sub>3</sub><sup>-</sup> can therefore lower apparent <sup>13</sup>C fractionation values. In addition, alterations in the CO<sub>2</sub> efflux over total carbon uptake (i.e., leakage) also affect <sup>13</sup>C fractionation, as faster replenishment of the intracellular CO<sub>2</sub> pool prevents a build-up of <sup>13</sup>CO<sub>2</sub> and thus allows RubisCO to fully express its intrinsic fractionation. Different modes of CCMs are employed by different phytoplankton species, likely



attributing to species-specific or group-specific differences in  $^{13}\text{C}$  fractionation (Badger et al., 1998; Van de Waal et al., 2019; Tortell, 2000).

70 The observed differences in  $\epsilon_p$  between nutrient-limited and nutrient-replete cultures have been attributed to differences in the regulation of carbon uptake relative to carbon fixation (Laws et al., 2001). This variation may, at least partly, be caused by culturing methods, as chemostat cultures that were limited by nutrients or light showed similar responses of  $\epsilon_p$  to changes in  $\text{CO}_2$ , while responses in light-controlled dilute batch cultures were markedly different (Laws et al., 2001). Likewise, discrepancies in measured  $\delta^{13}\text{C}$  values in different species of coccolithophores have been ascribed to varying culture methods, 75 in particular to methods of  $\text{CO}_2$  manipulation (Liu et al., 2018; Hermoso et al., 2016). A recent study furthermore attributed differences in apparent fractionation to a regulatory CCM pathway upstream of RubisCO (Wilkes and Pearson, 2019). This pathway was suggested to alleviate excess photon flux when cells are nutrient limited by shunting energy towards carbon uptake and hydroxylation reactions that increase  $\epsilon_p$ .

Here, we aim to elucidate which underlying physiological processes cause the observed differences in  $^{13}\text{C}$  fractionation in 80 phytoplankton under different  $\text{CO}_2$  concentrations, and how this is influenced by experimental settings. To this end, we collected data from all available culture studies, including a range of phytoplankton species from different groups, and evaluated systematic trends and offsets in  $^{13}\text{C}$  fractionation as a function of environmental, physiological, and experimental factors. This analysis compares the drivers behind phytoplankton  $^{13}\text{C}$  fractionation, assesses relations between  $^{13}\text{C}$  fractionation and culturing settings, and discusses implications for proxy development.

## 85 **2 Material & Methods**

### **2.1 Literature review**

We compiled data on  $^{13}\text{C}$  fractionation ( $\epsilon_p$ ) in phytoplankton species under a range of  $\text{CO}_2$  concentrations and experimental conditions. A literature search was performed in Web of Science (<https://www.webofknowledge.com/>) using the query (“phytoplankton” OR “algae” OR “microalgae” OR “picoplankton”) AND (“climate change” OR “ocean acidification” OR 90 “ $\text{CO}_2$ ” OR “carbon dioxide” OR “global change” OR “ $\text{pCO}_2$ ” OR “carbonate chemistry”) AND (“ $^{13}\text{C}$  fractionation” OR “ $\epsilon_p$ ” OR “carbon isotope” OR “isotope fractionation”) on 25-02-2020. Data on  $^{13}\text{C}$  fractionation, growth rates ( $\mu$ ), and particulate organic carbon (POC) content under different experimental conditions and  $\text{CO}_2$  concentrations were extracted using Engauge software when needed (Mitchell et al., 1991). To get an estimate of the carbon demands of the cells, we calculated POC production by multiplying the POC content with the instantaneous growth rate ( $\mu_i$ ). Using  $\mu_i$ , we yield POC production that 95 corresponds to the carbon fixation during the photoperiod (Riebesell et al., 2000b, a; Rost et al., 2002; Burkhardt et al., 1999a), and therefore corrects for difference in daylength between studies. In addition, information was extracted on experimental settings (i.e., irradiance, light-dark cycle, salinity, temperature, nutrients), culturing approach (i.e., batch, chemostat, dilute batch, dilute chemostat), and type of carbonate chemistry manipulation resulting in different concentrations of dissolved inorganic carbon (DIC; i.e., aeration of culture with  $\text{CO}_2$ , pre-aeration of culture medium with  $\text{CO}_2$ ) or total alkalinity (TA;



100 i.e., acid/base addition). Under non-limiting growth conditions,  $\delta^{13}\text{C}$  of phytoplankton cells was measured during the exponential growth phase. The database includes only marine and estuarine phytoplankton species, with data acquired through single species culture experiments.

## 2.2 Statistical analyses

105 All analyses were performed in R version 4.0.3 (R Core Team, 2020). Significant differences in  $\epsilon_p$  between different experimental conditions and culturing methods were calculated by means of a linear model followed by pairwise comparisons (Tukey method). To assess the relationship between POC production/ $\text{CO}_2$  and  $\epsilon_p$ , a linear model was fitted to the data for each of the distinct phytoplankton groups, and for each of the distinct species and study combinations. Data on POC production/ $\text{CO}_2$  was first log transformed, as this improved normality. To assess which of the influential conditions (i.e. nutrient conditions, carbonate chemistry manipulation method, culture approach, irradiance or light-dark cycle) could best explain the variation in  
110  $\epsilon_p$ , along with POC production/ $\text{CO}_2$ , we compared different models using the lmer function in R from the package “lme4” (Bates et al., 2015). In these models, POC production/ $\text{CO}_2$  and one of the influential conditions were fitted as fixed effects, including interaction terms, while species was fitted as a random effect for each of the distinct phytoplankton groups (excluding cyanobacteria due to lack of data). Models were subsequently compared based on their Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC).

## 115 3 Results

### 3.1 Dataset on $^{13}\text{C}$ fractionation

Our literature search yielded a total of 509 results, first titles and subsequently abstracts were reviewed, which led to a selection of 77 publications for screening. After careful screening for suitability, a total of 25 publications, containing 58 unique datasets, were included in our database. It contains data on four of the major marine phytoplankton groups, namely dinoflagellates (15  
120 datasets), diatoms (24 datasets), haptophytes (17 datasets) and cyanobacteria (2 datasets).

Across all phytoplankton groups, there is a negative log-linear relationship between  $^{13}\text{C}$  fractionation ( $\epsilon_p$ ) and POC production over  $\text{CO}_2$  supply (Fig. 1). This relationship is also apparent in each phytoplankton group (Fig. 2), although the slope of this curve varies between groups, and also strongly between species and studies (see also Fig. 3). As not all studies reported POC contents per cell, we also tested  $\mu_i/\text{CO}_2$  supply to assess more species (especially for diatoms), finding similar pattern as POC  
125 production/ $\text{CO}_2$  supply (Fig. S1).

### 3.1 Experimental settings and $^{13}\text{C}$ fractionation

Some of the variation in  $\epsilon_p$  can be explained by the different experimental settings between the studies (Fig. 4). For instance, phytoplankton grown under nitrogen limitation and lower temperatures show higher  $\epsilon_p$  than those grown under light-controlled or non-limiting growth conditions (Fig. 4d). However,  $^{13}\text{C}$  fractionation also varied across the different types of carbonate



130 chemistry manipulations and culturing approaches (Fig. 4a, c). Closed systems (i.e., pre-aeration with CO<sub>2</sub> and acid/base  
addition) had lower  $\epsilon_p$  values than open systems (continuous aeration with CO<sub>2</sub>), and cultures that were grown in chemostats  
with high biomass had higher overall  $\epsilon_p$  values than those grown in dilute cultures. Moreover, light-dark cycle also strongly  
influences <sup>13</sup>C fractionation, with cultures that experience continuous irradiation having higher  $\epsilon_p$  values than cultures that are  
exposed to a dark-cycle (Fig. 4b). This was especially apparent for haptophytes and dinoflagellates, where cultures with  
135 continuous light grown in nitrogen-limited chemostats had higher  $\epsilon_p$  values than those with a dark-cycle grown under replete  
dilute batch conditions (Fig. 2).

Some confounding experimental conditions across studies appear in our database. Notably, nutrient limitation experiments are  
almost always performed in chemostats with continuous aeration and without a light-dark cycle. In addition, non-limiting or  
light-controlled culture studies, with a light-dark cycle, are almost entirely performed using a dilute batch, with pre-aeration  
140 or acid/base addition (Fig. 4). To tease apart which of these confounding factors (i.e., nutrient conditions, type of carbonate  
chemistry manipulation, culturing approach, and light-dark cycle) can best explain the differences in  $\epsilon_p$ , besides C-demand/C-  
supply, we compared different models including POC production/CO<sub>2</sub> concentration and one of the variables for each of the  
distinct phytoplankton groups (Table S2-4). For haptophytes, inclusion of light-dark cycle could best explain the data (AIC  
711 and BIC 738), while culturing approach yielded the best results for dinoflagellates (AIC 490 and BIC 520), and for diatoms  
145 this was either culturing approach (AIC 600) or method of carbonate chemistry manipulation (BIC 623).

## 4 Discussion

In our analyses of the current literature on  $\epsilon_p$  responses, we observed a high dependence on C-demand/C-supply across and  
within different phytoplankton groups (Fig. 1, 2). This correction step for C-demand is essential, as already identified in  
previous work, because different growth rates and cellular C contents reflect the different C requirements of phytoplankton  
150 cells (Rau et al., 1996; Bidigare et al., 1997; Hoins et al., 2016). Variation in the  $\epsilon_p$  relationship with POC production/CO<sub>2</sub>  
was, however, observed between the different species and studies (Fig. 3). Next to species-specific differences, this may be  
attributed to the contrasting experimental settings and culture methods. In the following, we will discuss the variation in  
fractionation patterns between the phytoplankton species and groups, highlighting the potential role of CCMs, how different  
experimental settings may result in isotopic disequilibria conditions, and the implications for CO<sub>2</sub> proxies based on carbon  
155 isotope fractionation.

### 4.1 Fractionation patterns and underlying processes

Across and within phytoplankton groups, the relationship between  $\epsilon_p$  and POC production/CO<sub>2</sub> follows a decay function (i.e.  
see untransformed data Fig. S2), which highlights the active role of CCMs in C uptake in all groups. If species relied on  
diffusive CO<sub>2</sub> uptake alone, a more linear relationship can be expected. The presence of CCMs is further supported by some  
of the low  $\epsilon_p$  signals (Fig. 1), indicating a higher contribution of HCO<sub>3</sub><sup>-</sup> to C fixation and/or decreased leakage. Intrinsic  
160 RubisCO fractionation values ( $\epsilon_f$ ) of 18‰ and 11‰ were measured in diatoms and haptophytes, respectively (Boller et al.,



2011, 2015).  $\epsilon_p$  values exceeding  $\epsilon_f$  for both of these groups, and possibly also for dinoflagellates, remain therefore puzzling and indicate fractionation steps occurring upstream of RubisCO (Wilkes and Pearson, 2019).

In cyanobacteria, CO<sub>2</sub> fixation by RubisCO takes place in the carboxysome, which is a distinct cellular compartment. The membrane of this compartment prevents diffusion of CO<sub>2</sub>, while it is permeable for HCO<sub>3</sub><sup>-</sup> which is converted to CO<sub>2</sub> via carboxysomal CA, thereby accumulating CO<sub>2</sub> in the vicinity of RubisCO (Espie and Kimber, 2011; Dou et al., 2008; Price et al., 2008). To prevent CO<sub>2</sub> efflux out of the cell, and likewise facilitate diffusive CO<sub>2</sub> uptake, cytosolic CO<sub>2</sub> is actively converted to HCO<sub>3</sub><sup>-</sup> by the NAD(P)H dehydrogenase (NDH) complex in the cytoplasm (Price et al., 2002; Maeda et al., 2002). It was proposed that these specific processes modify and in fact raise  $\epsilon_p$  values in cyanobacteria: A strong disequilibrium in the cytosol may, for instance, favor a unidirectional conversion of CO<sub>2</sub> to HCO<sub>3</sub><sup>-</sup> that would result in an additional fractionation step of at least ~13‰ (O’Leary et al., 1992), and potentially up to 20-33‰ (Zeebe and Wolf-Gladrow, 2001; Zeebe, 2014; Siegenthaler and Münnich, 1981; Clark and Lauriol, 1992). If this conversion step is furthermore mediated by NDH, this enzyme will likely discriminate against <sup>13</sup>C resulting in additional fractionation (Eichner et al., 2015). Overall, this ‘internal C cycling’ around NDH would yield higher  $\epsilon_p$  values than otherwise expected based on the CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> fluxes over the plasma membrane assuming equilibrium (Eichner et al., 2015; Sharkey and Berry, 1985).

Similar strategies may be present in the other algal groups that increase  $\epsilon_p$ . Effective CO<sub>2</sub> fixation in diatoms relies on biophysical CCMs that facilitate or actively transport CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> through a four-layered chloroplast membrane system (Keeling, 2013), which principally make the uptake more challenging but also confer additional control on the DIC fluxes (Matsuda et al., 2017; Nakajima et al., 2013). Numerous subcellular localized CAs are present in diatoms, which accelerate the interconversion of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>, also within the pyrenoid, where RubisCO is localized (Tachibana et al., 2011; Kikutani et al., 2016; Samukawa et al., 2014). Chemical disequilibrium environments between compartments, unidirectional conversion of CAs, or <sup>13</sup>C discrimination associated with HCO<sub>3</sub><sup>-</sup> transporters (solute carrier type transporters; SLC) may represent additional, but likely small sources of <sup>13</sup>C fractionation for diatoms.

No internal membrane systems with localized CAs associated to C fixation as present in diatoms have been recognized in haptophytes and dinoflagellates (Rokitta et al., 2022). In fact, some dinoflagellate species even lack the pyrenoid compartment, where RubisCO is located in most eukaryotic algae (Ratti et al., 2007). The contribution of HCO<sub>3</sub><sup>-</sup> to photosynthesis is high in both groups (Rost et al., 2006; Rokitta and Rost, 2012; Bach et al., 2013; McClelland et al., 2017), and fractionation due to chemical disequilibria within the cell can therefore occur to some degree, e.g. by favoring unidirectional conversion of CO<sub>2</sub> to HCO<sub>3</sub><sup>-</sup> and vice versa. However, stronger internal C-cycling to maintain high CO<sub>2</sub> accumulation in proximity of RubisCO by decreasing CO<sub>2</sub> leakage from the cell (Cassar et al., 2006; Schulz et al., 2007; Eichner et al., 2015; Hoins et al., 2016) and higher contribution of HCO<sub>3</sub><sup>-</sup> to net C fixation generally lead to higher build-up of <sup>13</sup>C within the cell (i.e., stronger internal Rayleigh fractionation) and consequently lower  $\epsilon_p$  values. Thus, while described modes of CCMs for the different groups are mostly in line with observed fractionation patterns (Schulz et al., 2007; Eichner et al., 2015; Hoins et al., 2016; McClelland et al., 2017),  $\epsilon_p$  values exceeding the intrinsic fractionation of RubisCO remain puzzling.



195 Wilkes and Pearson (2019) recently proposed that certain components of the CCM are differently regulated in nutrient-limited, light-replete cultures compared to light-controlled cultures, which could explain the often observed differences in  $\epsilon_p$  patterns between chemostat and dilute batch cultures (Fig. 4), and likewise reconcile why  $\epsilon_p$  values can exceed  $\epsilon_f$  estimates under some conditions. More specifically, the authors suggested that when cells are nutrient limited they can experience excess photon flux, which may be alleviated through fueling photocatalytic dehydration reactions of  $\text{HCO}_3^-$  by internal CAs localized in the thylakoid lumen. The acidic environment in the thylakoid favors the unidirectional conversion of  $\text{HCO}_3^-$  to  $\text{CO}_2$ , while the alkaline environment in the chloroplast favors the unidirectional conversion of  $\text{CO}_2$  to  $\text{HCO}_3^-$ . Light-induced stimulation of these processes may increase fractionation due to unidirectional hydration of  $\text{CO}_2$  and dehydration of  $\text{HCO}_3^-$  (up to ~25‰ and ~34‰, respectively; Wilkes and Pearson, 2019). However, the proposed  $\epsilon_p$  difference between light-limited and nutrient-limited cultures was not consistently found (Fig 4; Laws et al., 2001; Hoins et al., 2016a). Moreover, our results suggest this “light-driven CCM” activity stems from the absence of a light-dark cycle during culture growth rather than from nutrient or light limitation (Fig. 1, 4b). This was especially the case for haptophytes and dinoflagellates, where  $\epsilon_p$  values were consistently elevated under continuous irradiance (Fig. 2). In addition to differences in light-dark cycle, other culturing variables also differed between the studies reviewed by Wilkes and Pearson (2019).

#### 4.2 Experimental settings and resulting isotopic disequilibria

210 Studies yielding exceptionally high  $\epsilon_p$  values (apparently higher than  $\epsilon_f$ ) were, next to nutrient limitation and continuous irradiance, also performed in high biomass chemostats under continuous aeration with  $\text{CO}_2$  (Fig. 4). In haptophytes, light-dark cycle could, next to POC production/ $\text{CO}_2$ , best explain differences in  $\epsilon_p$  (Table S2). The important role of light on fractionation was already discussed by Rost et al. (2002), and more recently highlighted by Phelps et al. (2021). They found that in coccolithophores,  $\epsilon_p$  depended more strongly on light intensity and light-dark cycle than on  $\text{CO}_2$  concentrations (higher  $\epsilon_p$  values with more light exposure), even when corrected for C demands (Phelps et al., 2021; Rost et al., 2002). While continuous light led to higher  $\epsilon_p$  also in one dinoflagellate and several diatoms (Burkhardt et al., 1999b; Wilkes et al., 2017), the light-dependency of  $\epsilon_p$  seems strongest in haptophytes and may thus relate to changes in C flow between photosynthetic C fixation and calcification under changing light conditions (Krumhardt et al., 2017; Bolton and Stoll, 2013; Phelps et al., 2021). For instance, daylength had a significant influence on  $\epsilon_p$  of *E. huxleyi* (up to 8‰), as the preferred carbon source shifted from  $\text{CO}_2$  under continuous light to  $\text{HCO}_3^-$  uptake under light-dark cycles (Rost et al., 2002; Rost et al., 2006). However, not all phytoplankton species’ fractionation responds similarly to changes in daylength. For two diatoms and one dinoflagellate species, for instance,  $\epsilon_p$  values were similar for cultures grown under continuous light or light-dark cycles (Burkhardt et al., 1999b).

In diatoms and dinoflagellates, the culturing approach or method of carbonate chemistry manipulation was, next to POC production/ $\text{CO}_2$ , the best predictor for changes in  $\epsilon_p$  (Table S3, S4). Rost et al. (2008) pointed out that, next to aspects of the CCM itself, different carbonate chemistry manipulations and culturing methods can lead to different  $\text{CO}_2$ -dependencies between studies and different experimental setups.



Importantly, in the calculations for the  $\delta^{13}\text{C}$  of  $\text{CO}_2$  and thus also  $\epsilon_p$ , chemical and isotopic equilibrium is assumed. In “open” carbonate chemistry systems with a continuous supply of  $\text{CO}_2$ , however, an equilibrium situation may not yet be reached before  
230 phytoplankton assimilate carbon (Zeebe et al., 1999; Rost et al., 2008), which is even more so in case of high biomass cultures. Recent work from Zhang et al. (2022) showed that it takes much longer (several hours to days) for an isotopic equilibrium to be reached in empty algal culturing vessels than a chemical equilibrium and that this should be considered in  $\epsilon_p$  calculations. This discrepancy could lead to an overestimation of  $\epsilon_p$  in “open” carbonate chemistry systems compared to “closed” carbonate chemistry systems, which has been setup by pre-aeration with a certain  $p\text{CO}_2$  or by acid/base additions, such as observed in  
235 figure 4c. This may especially be true for cultures with high  $\text{CO}_2$  treatments (high carbon supply) and high overall carbon demands (high biomass), as both favor disequilibria situations (see Fig. S3 for example of the dinoflagellate *Alexandrium tamarense*). In chemostats that were run with low cell densities, on the other hand, isotopic disequilibria may not play a role and therefore yield similar  $\epsilon_p$  values comparable to dilute batch studies (Fig. 4c). Hence, biases in  $\epsilon_p$  values introduced by isotopic disequilibria can be misinterpreted as treatment effects, e.g., as an effect of nitrate- vs light-limited growth. Moreover,  
240 chemostat systems that are maintained with high biomass, even though they are meant to mimic oligotrophic systems, are not representative for natural environments as these system support only low biomass concentrations (Van de Waal et al., 2014).

#### 4.3 Implications for proxies

The extent to which experiments reflect natural conditions is important regarding proxy development, as they feed the mechanistic model of  $\text{CO}_2$ -dependent carbon isotope fractionation and confounding factors. A more standardized approach in  
245 performing these types of experiments, so representative natural light settings, using only one type of carbonate chemistry manipulation, and maintaining cultures at low biomass, would already substantially reduce variation in the  $\text{CO}_2$ -dependent  $\epsilon_p$  responses between studies (Fig. 3). Both species-specific differences and the effects of drivers (nutrient limitation, temperature conditions, etc.) would then be more straightforward to distinguish, as study design will not interfere as a concomitant source of  $\epsilon_p$  variation (Fig. 4). We note, however, that even when studies use comparable methods, findings can still vary. For example,  
250 not all phytoplankton strains tested showed a negative relationship between  $\epsilon_p$  and POC production/ $\text{CO}_2$  (Fig. 3), meaning that other environmental factors can mask the  $\text{CO}_2$  dependence, which urges for caution when using  $^{13}\text{C}$  fractionation during photosynthetic carbon fixation as a  $\text{CO}_2$  proxy. Quantitative constraints on these confounding factors are crucial to guarantee that reconstructed signals exceed the related uncertainties.

Quantitative constraints on physiological variables, implied to be growth rate and cell geometry, but also membrane  
255 permeability to  $\text{CO}_2$ , and the boundary layer thickness dependent on temperature, pH, and salinity, are in place in  $\text{CO}_2$  proxy work with a catch-all term called  $b$  (Popp et al., 1998; Rau et al., 1996; Jasper and Hayes, 1990; Bolton et al., 2016; Stoll et al., 2019). The  $b$  value is often linearly correlated with modern dissolved reactive phosphate concentration in surface seawater (Bice et al., 2006; Bidigare et al., 1997; Pagani et al., 2005), as phosphate is a major nutrient that often co-limits with other important micronutrients such as iron, zinc, and cobalt, which affect phytoplankton growth rate and cell size (Bidigare et al.,



260 1997). However, this is quite a simplistic view and  $b$  can vary substantially over time and between locations (Zhang et al. 2019).

Better constraints on  $b$  may further advance CO<sub>2</sub> proxy development based on specific algal biomarkers, although this remains a challenge due to the sparsity of useful parameters on confounding factors in the paleo-environment. This is especially true for biomarker proxies, such as phytane and alkenones, as these biomarkers are produced by multiple species, certainly through geological time (Witkowski et al., 2018). These species may have had different modes of carbon acquisition, growth rates and cellular carbon contents, and discrepancies between alkenone proxy and ice core records were also attributed to CCM activity (Badger et al., 2019; Badger, 2020). The preference for more specific proxy work is also needed as the analyzed phytoplankton groups show different slopes for the  $\epsilon_p$  versus POC production/CO<sub>2</sub> relationship (Fig. 2). However, a better selection of study sites (i.e., located in more productive ocean regions with possibly similarly responsive species as well) can reproduce  $p\text{CO}_2$  estimates that are in agreement with the ice core records even with constant  $b$  values (Zhang et al., 2019).

Another phytoplankton group and even species-specific line of CO<sub>2</sub> proxies under development is the <sup>13</sup>C fractionation of dinoflagellate resting cysts (Hoins et al., 2015; Sluijs et al., 2018). Single cysts can be analyzed with a recent analytical setup (van Roij et al., 2017), which provides the advantage that specific species can be selected so that an estimate of cell size can be made. Vegetative cell sizes of dinoflagellates generally correspond to sizes of their resting cysts (Finkel et al., 2007). Cyst size may be used to infer cellular carbon contents, and together with phosphate concentrations for growth rate, can give a better estimate for carbon demands and therefore improve the constraints of  $b$  in this line of proxies.

## 5 Conclusions

Our results illustrate that the POC production/CO<sub>2</sub>-dependency of  $\epsilon_p$  can vary significantly between different phytoplankton species and groups, but also as a result of different culturing methods and differences in daylength, especially for haptophytes. Extrapolating results to natural environments and for proxy applications therefore requires caution, and it should be carefully considered if culture methods and experimental conditions are representative of natural environments. Better approximations for carbon demands (described by  $\mu_i$ ) in  $\epsilon_p$ -based CO<sub>2</sub> proxies could also greatly improve their estimates. This will be challenging in the paleo-environment, especially with proxies that rely on biomarkers. Alternatively, careful selection of sites with more similar environments and phytoplankton species could also further improve proxy estimates.

## 285 Data availability

All data presented in this study is available in the open data repository Dryad (DOI##)



### Author contributions

All authors contributed to the study design. Preliminary data for the study was collected by MH. KB collected additional data, performed statistical analyses and wrote the first draft. All authors provided feedback on the manuscript.

### 290 Competing interest

The authors declare that they have no conflict of interest.

### Acknowledgement

The authors thank Gert-Jan Reichart for constructive discussions that helped shape the presented ideas. AS thanks the European Research Council for Consolidator Grant 771497.

### 295 References

- Bach, L. T., MacKinder, L. C. M., Schulz, K. G., Wheeler, G., Schroeder, D. C., Brownlee, C., and Riebesell, U.: Dissecting the impact of CO<sub>2</sub> and pH on the mechanisms of photosynthesis and calcification in the coccolithophore *Emiliana huxleyi*, *New Phytol.*, 199, 121–134, 2013.
- Badger, M. P. S.: Alkenone isotopes show evidence of active carbon concentrating mechanisms in coccolithophores as aqueous carbon dioxide concentrations fall below 7 μmolL<sup>-1</sup>, 2, 1–19, 2020.
- 300 Badger, M. P. S., Chalk, T. B., Foster, G. L., Bown, P. R., Gibbs, S. J., Sexton, P. F., Schmidt, D. N., Pälike, H., Mackensen, A., and Pancost, R. D.: Insensitivity of alkenone carbon isotopes to atmospheric CO<sub>2</sub> at low to moderate CO<sub>2</sub> levels, *Clim. Past*, 15, 539–554, 2019.
- Badger, M. R., Andrews, T. J., Whitney, S. M., Ludwig, M., Yellowlees, D. C., Leggat, W., and Price, G. D.: The diversity and coevolution of Rubisco, plastids, pyrenoids, and chloroplast-based CO<sub>2</sub>-concentrating mechanisms in algae, *Can. J. Bot.*, 305 76, 1052–1071, 1998.
- Bates, D., Machler, M., Bolker, B., and Walker, S.: Fitting linear mixed-effects models using lme4, *J. Stat. Softw.*, 67, 1–48, 2015.
- Bice, K. L., Birgel, D., Meyers, P. A., Dahl, K. A., Hinrichs, K. U., and Norris, R. D.: A multiple proxy and model study of 310 Cretaceous upper ocean temperatures and atmospheric CO<sub>2</sub> concentrations, *Paleoceanography*, 21, 1–17, 2006.
- Bidigare, R., Freeman, H., Hanson, L., Hayes, M., Jasper, P., King, L., Millero, J., Popp, N., Steinberg, A., and Wakeham, G.: Consistent fractionation of <sup>13</sup>C in nature and in the laboratory: growth-rate effects in some haptophyte algae, *Global Biogeochem. Cycles*, 11, 279–292, 1997.
- Boller, A. J., Thomas, P. J., Cavanaugh, C. M., and Scott, K. M.: Low stable carbon isotope fractionation by coccolithophore



- 315 RubisCO, *Geochim. Cosmochim. Acta*, 75, 7200–7207, 2011.  
Boller, A. J., Thomas, P. J., Cavanaugh, C. M., and Scott, K. M.: Isotopic discrimination and kinetic parameters of RubisCO from the marine bloom-forming diatom, *Skeletonema costatum*, *Geobiology*, 13, 33–43, 2015.  
Bolton, C. T. and Stoll, H. M.: Late Miocene threshold response of marine algae to carbon dioxide limitation, *Nature*, 500, 558–562, 2013.
- 320 Bolton, C. T., Hernández-Sánchez, M. T., Fuertes, M. Á., González-Lemos, S., Abrevaya, L., Mendez-Vicente, A., Flores, J. A., Probert, I., Giosan, L., Johnson, J., and Stoll, H. M.: Decrease in coccolithophore calcification and CO<sub>2</sub> since the middle Miocene, *Nat. Commun.*, 7, 2016.  
Burkhardt, S., Riebesell, U., and Zondervan, I.: Effects of growth rate, CO<sub>2</sub> concentration, and cell size on the stable carbon isotope fractionation in marine phytoplankton, *Geochim. Cosmochim. Acta*, 63, 3729–3741, 1999a.
- 325 Burkhardt, S., Riebesell, U., and Zondervan, I.: Stable carbon isotope fractionation by marine phytoplankton in response to daylength, growth rate, and CO<sub>2</sub> availability, *Mar. Ecol. Prog. Ser.*, 184, 31–41, 1999b.  
Cassar, N., Laws, E. A., and Popp, B. N.: Carbon isotopic fractionation by the marine diatom *Phaeodactylum tricorutum* under nutrient- and light-limited growth conditions, *Geochim. Cosmochim. Acta*, 70, 5323–5335, 2006.  
Clark, I. D. and Lauriol, B.: Kinetic enrichment of stable isotopes in cryogenic calcites, *Chem. Geol.*, 102, 217–228, 1992.
- 330 Dou, Z., Heinhorst, S., Williams, E. B., Murin, C. D., Shively, J. M., and Cannon, G. C.: CO<sub>2</sub> fixation kinetics of *Halothiobacillus neapolitanus* mutant carboxysomes lacking carbonic anhydrase suggest the shell acts as a diffusional barrier for CO<sub>2</sub>, *J. Biol. Chem.*, 283, 10377–10384, 2008.  
Eichner, M., Thoms, S., Kranz, S. A., and Rost, B.: Cellular inorganic carbon fluxes in *Trichodesmium*: A combined approach using measurements and modelling, *J. Exp. Bot.*, 66, 749–759, 2015.
- 335 Espie, G. S. and Kimber, M. S.: Carboxysomes: cyanobacterial RubisCO comes in small packages, *Photosynth. Res.*, 109, 7–20, 2011.  
Farquhar, G. D., Ehleringer, J. R., and Hubick, K. T.: Carbon isotope discrimination and photosynthesis, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 40, 503–537, 1989.  
Finkel, Z. V., Sebbo, J., Feist-Burkhardt, S., Irwin, A. J., Katz, M. E., Schofield, O. M. E., Young, J. R., and Falkowski, P. G.:  
340 A universal driver of macroevolutionary change in the size of marine phytoplankton over the Cenozoic, *Proc. Natl. Acad. Sci. U. S. A.*, 104, 20416–20420, 2007.  
Foster, G. L., Royer, D. L., and Lunt, D. J.: Future climate forcing potentially without precedent in the last 420 million years, *Nat. Commun.*, 8, 1–8, 2017.  
Freeman, H. and Hayes, J. M.: Fractionation of carbon isotopes by phytoplankton and estimates of ancient CO<sub>2</sub> levels, *Global*  
345 *Biogeochem. Cycles*, 6, 185–198, 1992.  
Giordano, M., Beardall, J., and Raven, J. A.: CO<sub>2</sub> concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution, *Annu. Rev. Plant Biol.*, 56, 99–131, 2005.  
Hayes, J. M., Strauss, H., and Kaufman, A. J.: The abundance of <sup>13</sup>C in marine organic matter and isotopic fractionation in the



- global biogeochemical cycle of carbon during the past 800 Ma, *Chem. Geol.*, 161, 103–125, 1999.
- 350 Hermoso, M., Chan, I. Z. X., McClelland, H. L. O., Heureux, A. M. C., and Rickaby, R. E. M.: Vanishing coccolith vital effects with alleviated carbon limitation, *13*, 301–312, 2016.
- Hoins, M., Van de Waal, D. B., Eberlein, T., Reichart, G. J., Rost, B., and Sluijs, A.: Stable carbon isotope fractionation of organic cyst-forming dinoflagellates: evaluating the potential for a CO<sub>2</sub> proxy, *Geochim. Cosmochim. Acta*, 160, 267–276, 2015.
- 355 Hoins, M., Eberlein, T., Van de Waal, D. B., Sluijs, A., Reichart, G. J., and Rost, B.: CO<sub>2</sub>-dependent carbon isotope fractionation in dinoflagellates relates to their inorganic carbon fluxes, *J. Exp. Mar. Bio. Ecol.*, 481, 9–14, 2016a.
- Hoins, M., Eberlein, T., Großmann, C. H., Brandenburg, K., Reichart, G.-J., Rost, B., Sluijs, A., and Van De Waal, D. B.: Combined effects of ocean acidification and light or nitrogen availabilities on <sup>13</sup>C fractionation in marine dinoflagellates, *PLoS One*, 11, 2016b.
- 360 Jasper, J. P. and Hayes, J. M.: A carbon isotope record of CO<sub>2</sub> levels during the late Quaternary, *Nature*, 347, 462–464, 1990.
- Keeling, P. J.: The number, speed, and impact of plastid endosymbioses in eukaryotic evolution, *Annu. Rev. Plant Biol.*, 64, 583–607, 2013.
- Kikutani, S., Nakajima, K., Nagasato, C., Tsuji, Y., Miyatake, A., and Matsuda, Y.: Thylakoid luminal  $\Theta$ -carbonic anhydrase critical for growth and photosynthesis in the marine diatom *Phaeodactylum tricornutum*, *Proc. Natl. Acad. Sci. U. S. A.*, 113, 9828–9833, 2016.
- 365 Krumhardt, K. M., Lovenduski, N. S., Iglesias-Rodriguez, M. D., and Kleypas, J. A.: Coccolithophore growth and calcification in a changing ocean, *Prog. Oceanogr.*, 159, 276–295, 2017.
- Laws, E. A., Popp, B. N., Bidigare, R. R., Riebesell, U., Burkhardt, S., and Wakeham, S. G.: Controls on the molecular distribution and carbon isotopic composition of alkenones in certain haptophyte algae, *Geochemistry, Geophys. Geosystems*, 2, 2001.
- 370 Liu, Y. W., Eagle, R. A., Aciego, S. M., Gilmore, R. E., and Ries, J. B.: A coastal coccolithophore maintains pH homeostasis and switches carbon sources in response to ocean acidification, *Nat. Commun.*, 9, 1–12, 2018.
- Maberly, S. C., Raven, J. A., and Johnston, A. M.: Discrimination between <sup>12</sup>C and <sup>13</sup>C by marine plants, *Oecologia*, 91, 481–492, 1992.
- 375 Macdonald, F. A.: Deep-time paleoclimate proxies, *AGU Adv.*, 1, 1–3, 2020.
- Maeda, S. I., Badger, M. R., and Price, G. D.: Novel gene products associated with NdhD3/D4-containing NDH-1 complexes are involved in photosynthetic CO<sub>2</sub> hydration in the cyanobacterium, *Synechococcus* sp. PCC7942, *Mol. Microbiol.*, 43, 425–435, 2002.
- Matsuda, Y., Hopkinson, B. M., Nakajima, K., Dupont, C. L., and Tsuji, Y.: Mechanisms of carbon dioxide acquisition and CO<sub>2</sub> sensing in marine diatoms: a gateway to carbon metabolism, *Philos. Trans. R. Soc. B Biol. Sci.*, 372, 2017.
- 380 McClelland, H. L. O., Bruggeman, J., Hermoso, M., and Rickaby, R. E. M.: The origin of carbon isotope vital effects in coccolith calcite, *Nat. Commun.*, 8, 2017.



- McNevin, D. B., Badger, M. R., Whitney, S. M., Von Caemmerer, S., Tcherkez, G. G. B., and Farquhar, G. D.: Differences in carbon isotope discrimination of three variants of D-ribulose-1,5-bisphosphate carboxylase/oxygenase reflect differences in their catalytic mechanisms, *J. Biol. Chem.*, 282, 36068–36076, 2007.
- Mitchell, M., Muftakhidinov, B., and Winchen, T.: Engauge Digitizer 4.1, 1991.
- Nakajima, K., Tanaka, A., and Matsuda, Y.: SLC4 family transporters in a marine diatom directly pump bicarbonate from seawater, *Proc. Natl. Acad. Sci. U. S. A.*, 110, 1767–1772, 2013.
- O’Leary, M. H.: Measurement of the isotope fractionation associated with diffusion of carbon dioxide in aqueous solution, *J. Phys. Chem.*, 88, 823–825, 1984.
- O’Leary, M. H., Madhavan, S., and Paneth, P.: Physical and chemical basis of carbon isotope fractionation in plants, *Plant. Cell Environ.*, 15, 1099–1104, 1992.
- Pagani, M., Zachos, J. C., Freeman, K. H., Tipple, B., and Bohaty, S.: Atmospheric science: marked decline in atmospheric carbon dioxide concentrations during the Paleogene, *Science* (80-. ), 309, 600–603, 2005.
- Phelps, S. R., Hennon, G. M. M., Dyhrman, S. T., Hernández Limón, M. D., Williamson, O. M., and Polissar, P. J.: Carbon isotope fractionation in Noelaerhabdaceae algae in culture and a critical evaluation of the alkenone paleobarometer, *Geochemistry, Geophys. Geosystems*, 22, 1–20, 2021.
- Popp, B. N., Laws, E. A., Bidigare, R. R., Dore, J. E., Hanson, K. L., and Wakeham, S. G.: Effect of phytoplankton cell geometry on carbon isotopic fractionation, *Geochim. Cosmochim. Acta*, 62, 69–77, 1998.
- Price, G. D., Maeda, S. I., Omata, T., and Badger, M. R.: Modes of active inorganic carbon uptake in the cyanobacterium, *Synechococcus* sp. PCC7942, *Funct. Plant Biol.*, 29, 131–149, 2002.
- Price, G. D., Badger, M. R., Woodger, F. J., and Long, B. M.: Advances in understanding the cyanobacterial CO<sub>2</sub>-concentrating- mechanism (CCM): Functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants, *J. Exp. Bot.*, 59, 1441–1461, 2008.
- R Core, T.: R: A language and environment for statistical computing, 2020.
- Ratti, S., Giordano, M., and Morse, D.: CO<sub>2</sub>-concentrating mechanisms of the potentially toxic dinoflagellate *Protoceratium reticulatum* (Dinophyceae, Gonyaulacales), *J. Phycol.*, 43, 693–701, 2007.
- Rau, G. H., Riebesell, U., and Wolf-Gladrow, D.: A model of photosynthetic <sup>13</sup>C fractionation by marine phytoplankton based on diffusive molecular CO<sub>2</sub> uptake, *Mar. Ecol. Prog. Ser.*, 133, 275–285, 1996.
- Raven, J. A. and Johnston, A.: Mechanisms of inorganic-carbon acquisition in marine phytoplankton and their implications for the use of other resources, *Limnol. Oceanogr.*, 36, 1701–1714, 1991.
- Reinfelder, J. R.: Carbon concentrating mechanisms in eukaryotic marine phytoplankton, *Ann. Rev. Mar. Sci.*, 3, 291–315, 2011.
- Riebesell, U., Burkhardt, S., Dauelsberg, A., and Kroon, B.: Carbon isotope fractionation by a marine diatom: dependence on the growth-rate-limiting resource, *Mar. Ecol. Prog. Ser.*, 193, 295–303, 2000a.
- Riebesell, U., Revill, A. T., Holdsworth, D. G., and Volkman, J. K.: The effects of varying CO<sub>2</sub> concentration on lipid



- composition and carbon isotope fractionation in *Emiliana huxleyi*, *Geochim. Cosmochim. Acta*, 64, 4179–4192, 2000b.
- Roeske, C. A. and O’Leary, M. H.: Carbon isotope effects on the enzyme-catalyzed carboxylation of ribulose biphosphate, *Biochemistry*, 23, 6275–6284, 1984.
- 420 Rohling, E. J., Sluijs, A., Dijkstra, H. A., Köhler, P., Van De Wal, R. S. W., Von Der Heydt, A. S., Beerling, D. J., Berger, A., Bijl, P. K., Crucifix, M., Deconto, R., Drijfhout, S. S., Fedorov, A., Foster, G. L., Ganopolski, A., Hansen, J., Hönlisch, B., Hooghiemstra, H., Huber, M., Huybers, P., Knutti, R., Lea, D. W., Lourens, L. J., Lunt, D., Masson-Demotte, V., Medina-Elizalde, M., Otto-Bliesner, B., Pagani, M., Pälike, H., Renssen, H., Royer, D. L., Siddall, M., Valdes, P., Zachos, J. C., and Zeebe, R. E.: Making sense of palaeoclimate sensitivity, *Nature*, 491, 683–691, <https://doi.org/10.1038/nature11574>, 2012.
- 425 van Roij, L., Sluijs, A., Laks, J. J., and Reichart, G. J.: Stable carbon isotope analyses of nanogram quantities of particulate organic carbon (pollen) with laser ablation nano combustion gas chromatography/isotope ratio mass spectrometry, *Rapid Commun. Mass Spectrom.*, 31, 47–58, 2017.
- Rokitta, S. D. and Rost, B.: Effects of CO<sub>2</sub> and their modulation by light in the life-cycle stages of the coccolithophore *Emiliana huxleyi*, *Limnol. Oceanogr.*, 57, 607–618, 2012.
- 430 Rokitta, S. D., Kranz, S. A., and Rost, B.: Inorganic carbon acquisition by aquatic primary producers, in: *Blue planet, red and green photosynthesis*, edited by: Maberly, S. and Gontero, B., ISTE Ltd, London, 2022.
- Rost, B., Zondervan, I., and Riebesell, U.: Light-dependent carbon isotope fractionation in the coccolithophorid *Emiliana huxleyi*, *Limnol. Oceanogr.*, 47, 120–128, 2002.
- Rost, B., Riebesell, U., and Sültemeyer, D.: Carbon acquisition of marine phytoplankton: effect of photoperiod length, *Limnol. Oceanogr.*, 51, 12–20, 2006.
- 435 Rost, B., Zondervan, I., and Wolf-Gladrow, D.: Sensitivity of phytoplankton to future changes in ocean carbonate chemistry: current knowledge, contradictions and research directions, *Mar. Ecol. Prog. Ser.*, 373, 227–237, 2008.
- Samukawa, M., Shen, C., Hopkinson, B. M., and Matsuda, Y.: Localization of putative carbonic anhydrases in the marine diatom, *Thalassiosira pseudonana*, *Photosynth. Res.*, 121, 235–249, 2014.
- 440 Schulz, K. G., Rost, B., Burkhardt, S., Riebesell, U., Thoms, S., and Wolf-Gladrow, D. A.: The effect of iron availability on the regulation of inorganic carbon acquisition in the coccolithophore *Emiliana huxleyi* and the significance of cellular compartmentation for stable carbon isotope fractionation, *Geochim. Cosmochim. Acta*, 71, 5301–5312, 2007.
- Sharkey, T. D. and Berry, J. A.: Carbon isotope fractionation of algae as influenced by an inducible CO<sub>2</sub> concentrating mechanism, in: *Inorganic Carbon Uptake by Aquatic Photosynthetic Organisms*, edited by: Lucas, W. J. and Berry, J. A., American Society of Plant Physiologists, 389–401, 1985.
- 445 Siegenthaler, U. and Münnich, K. O.: <sup>12</sup>C/<sup>13</sup>C fractionation during CO<sub>2</sub> transfer from air to sea, *Carbon Cycle Model.*, 249–251, 1981.
- Sluijs, A., van Roij, L., Frieling, J., Laks, J., and Reichart, G. J.: Single-species dinoflagellate cyst carbon isotope ecology across the Paleocene-Eocene Thermal Maximum, *Geology*, 46, 79–82, 2018.
- 450 Stoll, H. M., Guitian, J., Hernandez-Almeida, I., Mejia, L. M., Phelps, S., Polissar, P., Rosenthal, Y., Zhang, H., and Ziveri,



- P.: Upregulation of phytoplankton carbon concentrating mechanisms during low CO<sub>2</sub> glacial periods and implications for the phytoplankton pCO<sub>2</sub> proxy, *Quat. Sci. Rev.*, 208, 1–20, 2019.
- 455 Tabita, F. R., Satagopan, S., Hanson, T. E., Kreel, N. E., and Scott, S. S.: Distinct form I, II, III, and IV Rubisco proteins from the three kingdoms of life provide clues about Rubisco evolution and structure/function relationships, *J. Exp. Bot.*, 59, 1515–1524, 2008.
- Tachibana, M., Allen, A. E., Kikutani, S., Endo, Y., Bowler, C., and Matsuda, Y.: Localization of putative carbonic anhydrases in two marine diatoms, *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*, *Photosynth. Res.*, 109, 205–221, 2011.
- 460 Tierney, J. E., Poulsen, C. J., Montañez, I. P., Bhattacharya, T., Feng, R., Ford, H. L., Hönlisch, B., Inglis, G. N., Petersen, S. V., Sagoo, N., Tabor, C. R., Thirumalai, K., Zhu, J., Burls, N. J., Foster, G. L., Goddérís, Y., Huber, B. T., Ivany, L. C., Turner, S. K., Lunt, D. J., McElwain, J. C., Mills, B. J. W., Otto-Bliesner, B. L., Ridgwell, A., and Zhang, Y. G.: Past climates inform our future, *Science (80-. )*, 370, 2020.
- Tortell, P. D.: Evolutionary and ecological perspectives on carbon acquisition in phytoplankton, *Limnol. Oceanogr.*, 45, 744–750, 2000.
- 465 Van de Waal, D. B., Brandenburg, K. M., Keuskamp, J., Trimborn, S., Rokitta, S., Kranz, S., and Rost, B.: Highest plasticity of carbon concentrating mechanisms in earliest evolved phytoplankton, *Limnol. Oceanogr. Lett.*, 2019.
- Van De Waal, D. B., Eberlein, T., Bublitz, Y., John, U., and Rost, B.: Shake it easy: a gently mixed continuous culture system for dinoflagellates, *J. Plankton Res.*, 36, 889–894, 2014.
- Whitney, S. M., Houtz, R. L., and Alonso, H.: Advancing our understanding and capacity to engineer nature’s CO<sub>2</sub>-sequestering enzyme, Rubisco, *Plant Physiol.*, 155, 27–35, 2011.
- 470 Wilkes, E. B. and Pearson, A.: A general model for carbon isotopes in red-lineage phytoplankton: interplay between unidirectional processes and fractionation by RubisCO, *Geochim. Cosmochim. Acta*, 265, 163–181, 2019.
- Wilkes, E. B., Carter, S. J., and Pearson, A.: CO<sub>2</sub>-dependent carbon isotope fractionation in the dinoflagellate *Alexandrium tamarense*, *Geochim. Cosmochim. Acta*, 212, 48–61, 2017.
- 475 Witkowski, C. R., Weijers, J. W. H., Blais, B., Schouten, S., and Sinninghe Damsté, J. S.: Molecular fossils from phytoplankton reveal secular P<sub>CO2</sub> trend over the phanerozoic, *Sci. Adv.*, 4, 1–8, 2018.
- Zeebe, R. E.: Kinetic fractionation of carbon and oxygen isotopes during hydration of carbon dioxide, *Geochim. Cosmochim. Acta*, 139, 540–552, <https://doi.org/10.1016/j.gca.2014.05.005>, 2014.
- Zeebe, R. E. and Wolf-Gladrow, D. A.: CO<sub>2</sub> in Seawater: Equilibrium, Kinetics, Isotopes, Elsevier Science Publishing Co., Amsterdam, the Netherlands, 2001.
- 480 Zeebe, R. E., Wolf-Gladrow, D. A., and Jansen, H.: On the time required to establish chemical and isotopic equilibrium in the carbon dioxide system in seawater, *Mar. Chem.*, 65, 135–153, 1999.
- Zhang, H., Torres-Romero, I., Anjewierden, P., Jaggi, M., and Stoll, H. M.: The DIC carbon isotope evolutions during CO<sub>2</sub> bubbling: implications for ocean acidification laboratory culture, *Chem. Geol.*, 2022.
- Zhang, Y. G., Pearson, A., Benthien, A., Dong, L., Huybers, P., Liu, X., and Pagani, M.: Refining the alkenone-pCO<sub>2</sub> method



- 485 I: Lessons from the Quaternary glacial cycles, *Geochim. Cosmochim. Acta*, 260, 177–191, 2019.  
Zhu, J., Poulsen, C. J., and Otto-Bliesner, B. L.: High climate sensitivity in CMIP6 model not supported by paleoclimate, *Nat. Clim. Chang.*, 10, 378–379, 2020.

490

495

500

505

510

515



520 Figures

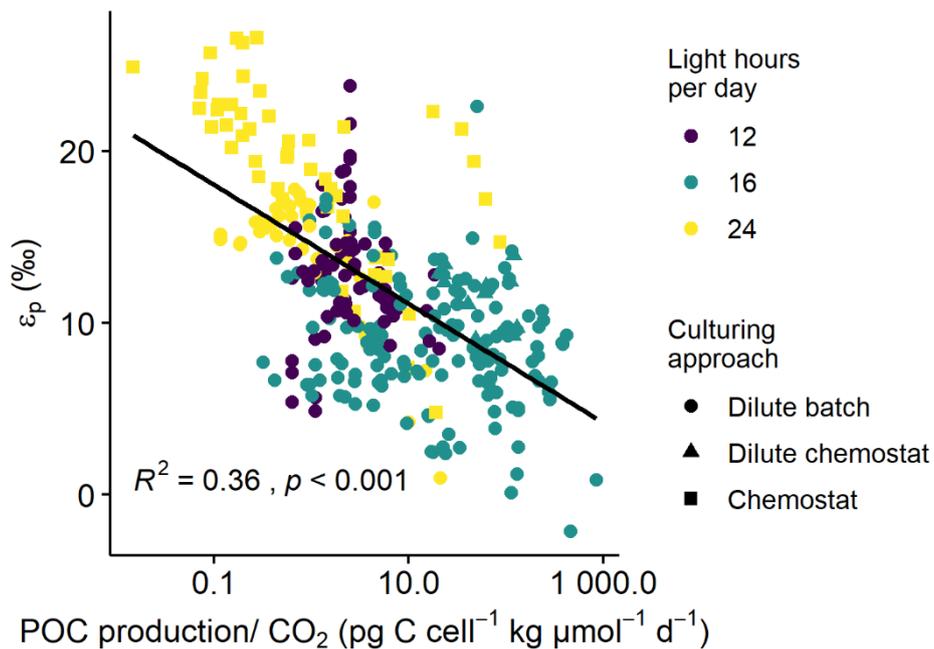
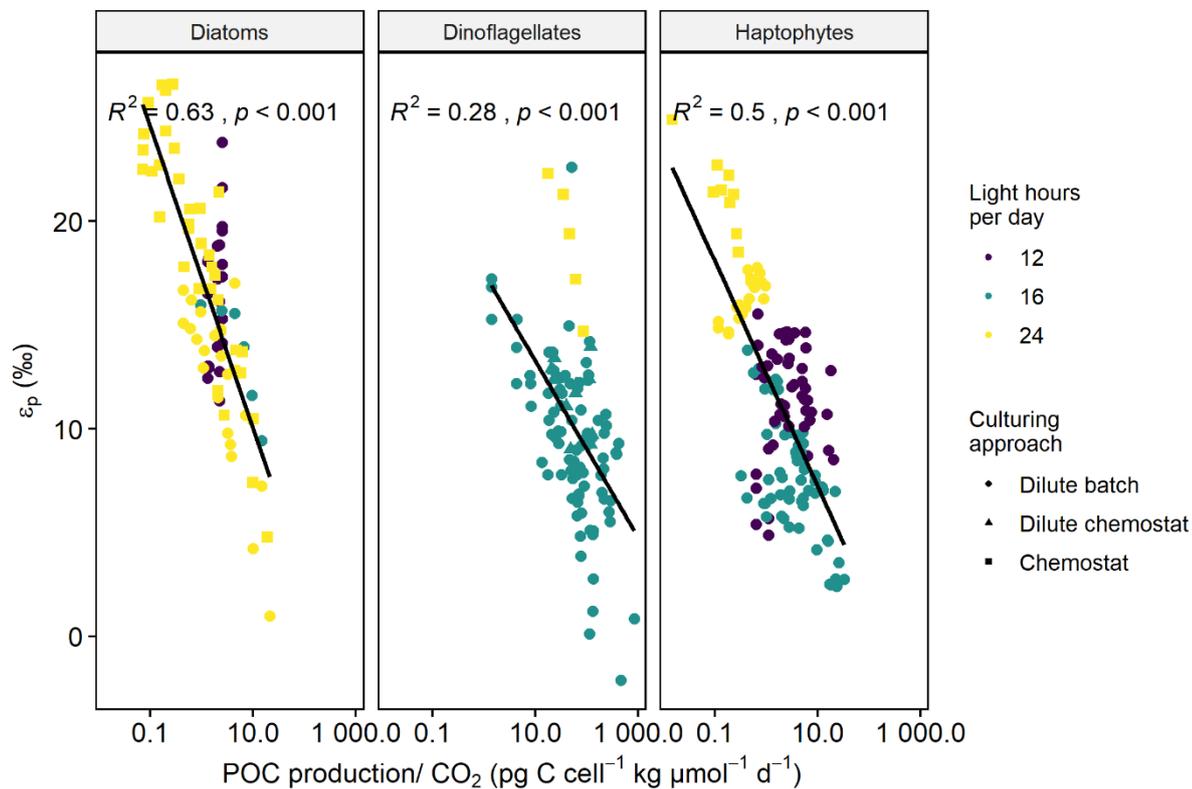
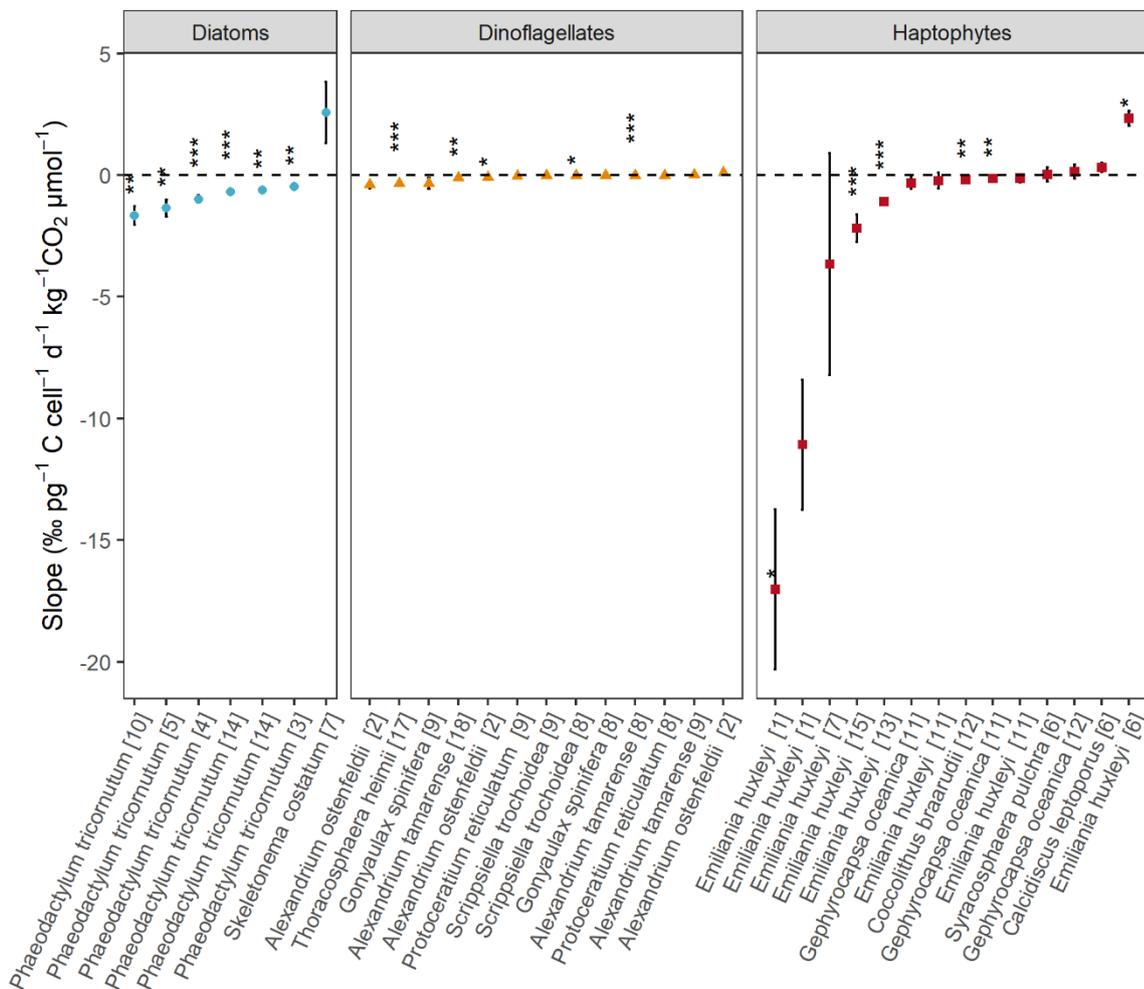


Figure 1: POC production/CO<sub>2</sub> (C-demand/C-supply; log-transformed) against ε<sub>p</sub> across all phytoplankton groups. Colors indicate the light-dark cycle; marker shapes indicate the culturing approach. Black line illustrates the log-linear relationship ( $R^2$  and  $P$  indicated in the panel).



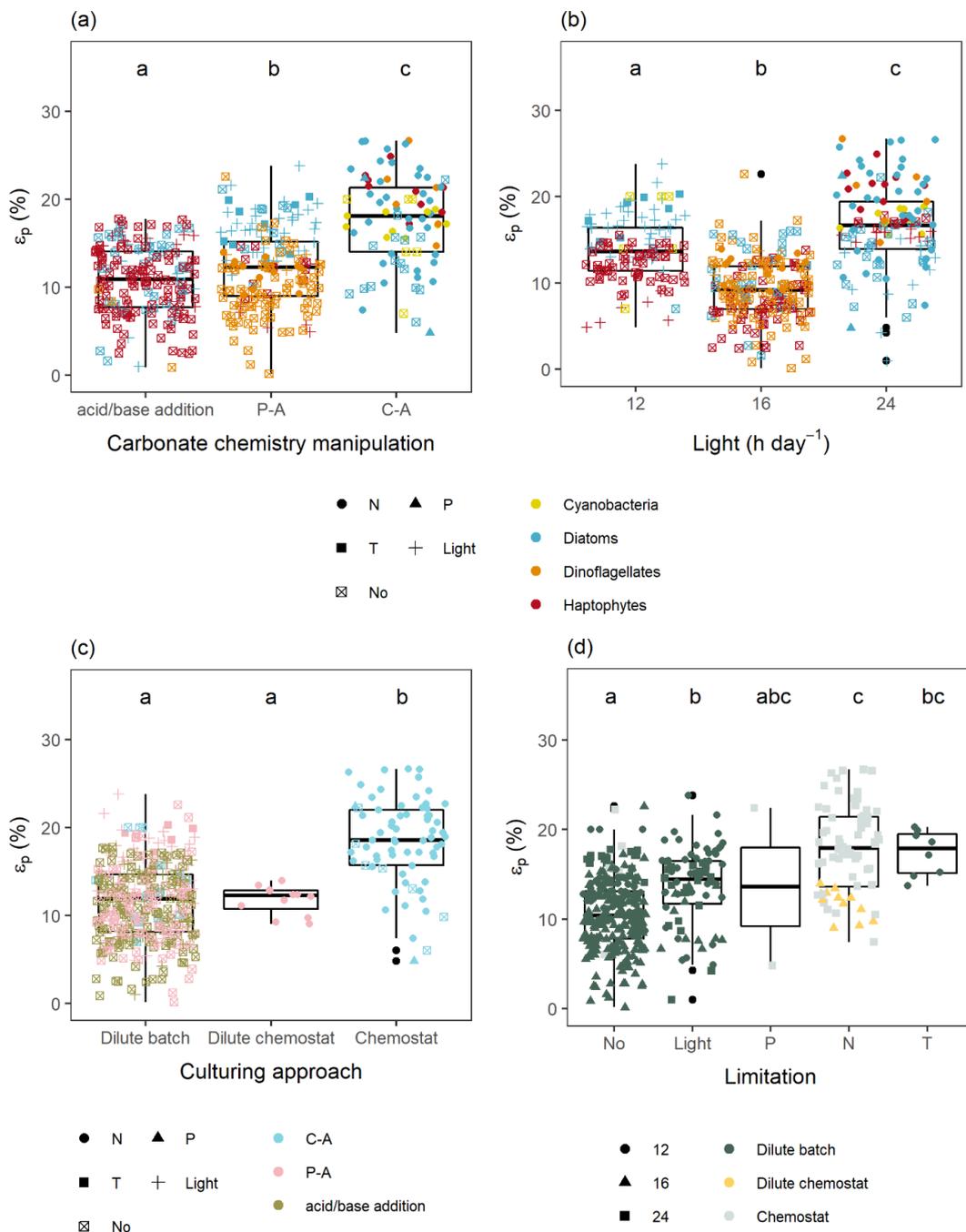
525

**Figure 2: POC production/CO<sub>2</sub> (carbon demand over supply; log-transformed) against  $\epsilon_p$  for the different phytoplankton groups, where the colored points indicate the respective light-dark cycle, and the shape of the points indicates the culturing approach. Black line illustrates the linear relationship ( $R^2$  and P indicated in the panels).**



530

**Figure 3: Slopes of  $\epsilon_p$  in response to POC production/ $\text{CO}_2$  for the different species and studies using a linear fit. Numbers between brackets refer to the different studies (Table S1). Blue dots represent diatoms, orange triangles dinoflagellates, and red squares haptophytes. Significance is indicated by the asterisks (\*\*\*)  $P < 0.001$ , \*\*  $P < 0.01$ , \*  $P < 0.05$ ).**



535

**Figure 4: Differences in  $\epsilon_p$  between a) type of carbonate chemistry manipulation (P-A is pre-aeration, and C-A is continuous aeration), b) light-dark cycle, where colors indicate the different phytoplankton groups and shapes indicate type of limitation (N is nitrogen, P is phosphorus, T is temperature, and No is non-limited), c) culturing approach, where colors indicate type of carbonate chemistry manipulations and shapes type of limitation again, and d) type of limitation, where colors indicate culturing approach and shapes light hours per day. Significant differences between experimental conditions are indicated by the letters.**

540