

## Review

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We dedicate this paper to the late Craig Sandgren who made major contributions to our understanding of the Chrysophyceae and Syurophyceae.

Acquisition and metabolism of carbon  
in the Ochrophyta other than diatomsJohn A. Raven<sup>1,2</sup> and Mario Giordano<sup>3,4</sup><sup>1</sup>Division of Plant Sciences, University of Dundee at the James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK<sup>2</sup>Climate Change Cluster, University of Technology Sydney, Ultimo, New South Wales 2007, Australia<sup>3</sup>Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona 60131, Italy<sup>4</sup>Institute of Microbiology, Academy of Sciences of the Czech Republic, Tréboň 37901, Czech Republic

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The acquisition and assimilation of inorganic C have been investigated in several of the 15 clades of the Ochrophyta other than diatoms, with biochemical, physiological and genomic data indicating significant mechanistic variation. Form ID Rubiscos in the Ochrophyta are characterized by a broad range of kinetics values. In spite of relatively high  $K_{0.5CO_2}$  and low  $CO_2 : O_2$  selectivity, diffusive entry of  $CO_2$  occurs in the Chrysophyceae and Synurophyceae. Eustigmatophyceae and Phaeophyceae, on the contrary, have  $CO_2$  concentrating mechanisms, usually involving the direct or indirect use of  $HCO_3^-$ . This variability is possibly due to the ecological contexts of the organism. In brown algae, C fixation generally takes place through a classical C3 metabolism, but there are some hints of the occurrence of C4 metabolism and low amplitude CAM in a few members of the Fucales. Genomic data show the presence of a number of potential C4 and CAM genes in Ochrophyta other than diatoms, but the other core functions of many of these genes give a very limited diagnostic value to their presence and are insufficient to conclude that C4 photosynthesis is present in these algae.

This article is part of the themed issue 'The peculiar carbon metabolism in diatoms'.

## 1. Introduction

Stramenopile eukaryotes comprise a diverse clade with a great range of nutritional modes [1] (table 1). Ochrophyta (i.e. Ochrista) is a diverse phylum of stramenopiles; most of the ochrophytan clades (16 in [5]) are photosynthetic or have photosynthetic members. The rest of the stramenopiles, the ecologically and economically very significant oomycetes and some others clades (a total of 8 in [2]), are all non-photosynthetic. The phylogenetic relations among these organisms are discussed by Baurain *et al.* [6], Brown & Sorhannus [7], Beakes *et al.* [2], Schmidt *et al.* [8] and Yang *et al.* [5].

The Bacillariophyceae is the best-investigated class of ochrophytes with respect to inorganic C acquisition and assimilation, showing the widespread occurrence of  $CO_2$  concentrating mechanisms (CCMs) and of pyrenoids, energized transport of  $HCO_3^-$  and limited support for C<sub>4</sub> or C<sub>4</sub>-like photosynthetic metabolism [9–12]. The emphasis on diatoms is reasonable in view of their phylogenetic and ecological diversity [13] and their contribution to global biogeochemical cycles (e.g. a marine diatom net primary productivity of about 20 Pg C per year [14]). However, there are 15 (possibly more) other ochrophyte classes with photosynthetic members [5–8,15]. The benthic marine macroalgal Phaeophyceae play a major role in primary productivity and as ecosystem engineers on rocky shores, especially in temperate and polar regions, with several species exceeding 10 m in length [16]. The Chrysophyceae and Synurophyceae are significant in the phytoplankton of acidic and often  $CO_2$ -enriched freshwaters [3,17–19], the microalga *Nannochloropsis* (Eustigmatophyceae) is being assessed for its biotechnological potential [20–22] and members of the Pelagophyceae and

**Table 1.** Examples of modes of nutrition in the Ochrophyta. See Raven *et al.* [1] for definition of modes of nutrition.

nutritional mode	example	references
free-living osmoorganotrophy	most Saprolegniales, e.g. <i>Saprolegnia</i> (oomycetes)	[2]
symbiotic (all parasitic) <sup>a</sup> osmoorganotrophy	perenosporalean clades, e.g. <i>Phytophthora</i> <sup>b</sup> (oomycetes); bicoecids; <i>Cafeteria</i>	[2]
phagoorganotrophy	some Chrysophyceae, e.g. <i>Paraphysomonas</i>	[2]
photolithotrophy	examples: Bacillariophyceae, Eustigmatophyceae, Phaeophyceae, Synurophyceae	[3]
phagophotomixotrophy	many Chrysophyceae	[3,4]

<sup>a</sup>Symbiosis is used in the broad sense, including mutualism and parasitism.

<sup>b</sup>Major parasite of flowering plants.

Raphidophyceae can form harmful algal blooms [23]. Here we synthesize what is known about inorganic carbon acquisition and assimilation in these non-diatom ochrophytes for comparison with what is known for diatoms.

## 2. Mechanisms of inorganic C influx: CO<sub>2</sub> diffusion and biophysical CO<sub>2</sub> concentrating mechanisms

Diffusive CO<sub>2</sub> entry from the bulk phase to ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) is driven by a concentration difference produced by the photosynthetic consumption of CO<sub>2</sub> that produces a lower CO<sub>2</sub> concentration in the proximity of Rubisco active sites than in the bulk phase supplying the CO<sub>2</sub>. Here the energization of the flux is indirect, coming from the use of absorbed photons for the reduction of NADP<sup>+</sup> to NADPH and the phosphorylation of ADP to ATP; the NADPH and ATP are then used in CO<sub>2</sub> assimilation producing, in the first instance, carbohydrate. Under these circumstances, the CO<sub>2</sub> concentration and the CO<sub>2</sub>:O<sub>2</sub> ratio at the Rubisco site typically allow significant Rubisco oxygenase activity and a corresponding operation of a photorespiratory C oxidation cycle that converts the 2-P-glycolate from Rubisco oxygenase into triose phosphate, with input of energy, directly or indirectly, from the thylakoid reactions of photosynthesis [24].

The functioning of CCMs, by definition, involves a higher steady state concentration of CO<sub>2</sub> at the site of Rubisco than occurs in the bulk medium. This transport of CO<sub>2</sub> against the free energy (concentration) gradient is energized independently of the energy input to the conversion of CO<sub>2</sub> to carbohydrate. This is the case for both the biophysical (energized, uphill transport of HCO<sub>3</sub><sup>-</sup> or CO<sub>2</sub> or H<sup>+</sup> across a cell membrane) and the biochemical (C4 photosynthesis or Crassulacean acid metabolism, CAM) CCMs. This parallel input of energy comes ultimately from the thylakoid reactions of photosynthesis, but sometimes indirectly via photosynthate production and its catabolism through glycolysis and the mitochondrial reactions [24]. Generally, despite the occurrence of CCMs, the CO<sub>2</sub>:O<sub>2</sub> ratio at the Rubisco active site is not higher enough to prevent a small flux of organic C through Rubisco oxygenase and a photorespiratory C oxidation cycle that adds to the energy cost [25], as does the inevitable leakage of some of the CO<sub>2</sub> accumulated at the Rubisco site [24,26,27].

## 3. Inorganic C acquisition in Ochrophyta other than diatoms

### (a) Introduction

There is substantial physiological evidence on the mechanism of inorganic carbon acquisition for a number of classes of the Ochrophyta, i.e. Chrysophyceae, Eustigmatophyceae, Phaeophyceae and Synurophyceae, with a little information available for a few other classes; genomic data are also available for the Eustigmatophyceae and the Phaeophyceae. We also discuss the occurrence of pyrenoids, showing that pyrenoids are not always required for stramenopile CCMs [9,28], and there can be pyrenoids but no obvious CCM activity [3].

A common rationale for the occurrence of diffusive CO<sub>2</sub> entry or of a CCM is the natural external CO<sub>2</sub> and O<sub>2</sub> concentrations and the kinetics and intracellular content of Rubisco. There is considerable variation in the kinetics, e.g. K<sub>0.5</sub> for CO<sub>2</sub>, K<sub>0.5</sub> for O<sub>2</sub> and CO<sub>2</sub>:O<sub>2</sub> selectivity, among the form ID Rubiscos in the Ochrophyta (table 2), as well as variations in the CO<sub>2</sub>-saturated rate of CO<sub>2</sub> fixation per gram of Rubisco protein, with high CO<sub>2</sub>-saturated specific reaction rates being generally associated with high K<sub>0.5</sub> for CO<sub>2</sub> and a low CO<sub>2</sub>:O<sub>2</sub> selectivity (= Srel or  $\tau$ ) [35]. However, significant variations of K<sub>0.5</sub>(CO<sub>2</sub>) and CO<sub>2</sub>:O<sub>2</sub> selectivity may occur within the mechanistic possibilities suggested by Tcherkez *et al.* [35]; this is the case for diatoms [29], in which there is evidence of positive selection of Rubisco [36]. Diffusive CO<sub>2</sub> entry is more likely in organisms whose Rubiscos have a relatively low K<sub>0.5</sub>(CO<sub>2</sub>) and a high CO<sub>2</sub>:O<sub>2</sub> selectivity; diffusive CO<sub>2</sub> entry would be particularly favoured if there is a CO<sub>2</sub> subsidy from the terrestrial catchment groundwater supply to rivers and some small lakes [37,38]. By contrast, Rubiscos with a high K<sub>0.5</sub>(CO<sub>2</sub>) and a low CO<sub>2</sub>:O<sub>2</sub> selectivity combined with no CO<sub>2</sub> subsidy and approximately air-equilibrium CO<sub>2</sub> and O<sub>2</sub> concentrations would be compatible with the occurrence of a CCM. The data in table 2 show that this expectation is not always met, with diffusive entry of CO<sub>2</sub> in the Chrysophyceae and Synurophyceae, and CCMs in the Eustigmatophyceae and Phaeophyceae (see below). The occurrence of diffusive CO<sub>2</sub> entry in organisms with relatively high Rubisco K<sub>0.5</sub>(CO<sub>2</sub>) could be rationalized by the availability of sufficient energy, N and P (for the RNA needed for protein synthesis) to produce more Rubisco per cell and/or a large CO<sub>2</sub> subsidy.

**Table 2.** *In vitro* kinetics of ochrophytan Rubiscos. n.d., not determined.

species	class	CCM	$K_{0.5}\text{CO}_2$ ( $\text{mmol m}^{-3}$ )	$K_{0.5}\text{O}_2$ ( $\text{mmol m}^{-3}$ )	$\text{CO}_2/\text{O}_2$ selectivity	references
11 species	Bacillariophyceae	+	23–68	413–2032	57–116	[29]
<i>Nannochloopsis</i> sp.	Eustigmatophyceae	+	7–10	about 1000	27	[30]
<i>Mallomonas papillosa</i>	Synurophyceae	–	18.2	n.d.	n.d.	[31]
<i>Synura petersenii</i>	Synurophyceae	–	28.4	n.d.	n.d.	[31]
<i>Synura uvella</i>	Synurophyceae	–	41.8	n.d.	n.d.	[31]
4 species	Phaeophyceae	+	12–43	n.d.	n.d.	[32]
<i>Olisthodiscus luteus</i>	Raphidophyceae	?	45–59	692	101	[9,33,34]

### (b) Chrysophyceae and Synurophyceae

The sister classes Chrysophyceae and Synurophyceae both have diffusive  $\text{CO}_2$  transport from the bulk medium to Rubisco. Most of the data relate to the Synurophyceae [3,17,31,39–42], with some for the Chrysophyceae [3,41], which, as indicated in table 1, are frequently phagomixotrophic [43].

The evidence on inorganic C entry includes pH drift. Through this method, it was possible to show that the photosynthetic activity of these algae can increase the pH and deplete  $\text{CO}_2$  to values attributable to a diffusive  $\text{CO}_2$  entry. More direct estimates of relatively high  $\text{CO}_2$  compensation concentrations from net photosynthetic rates as a function of inorganic C concentration, as well as estimates of photorespiration, support the view of inorganic carbon acquisition in Chrysophyceae and Synurophyceae as mostly due to  $\text{CO}_2$  diffusive entry driven by consumption of  $\text{CO}_2$  in the chloroplasts. For the two cases investigated (one Chrysophyceae and one Synurophyceae), there is no external carbonic anhydrase activity [3]. Vegetative cells of the Synurophyceae and cysts of the Chrysophyceae and Synurophyceae have extraprotoplasmic silicification, in common with several other clades of the Ochrophyta, especially the Bacillariophyceae and Bolidophyceae [44,45]. Among other roles of silicification is that as a pH buffer facilitating  $\text{H}^+$  movement related to extracellular carbonic anhydrase activity [46]; this cannot be the case in the Chrysophyceae and Synurophyceae with no extracellular carbonic anhydrase. Despite the lack of CCMs, pyrenoids occur in some Chrysophyceae (*Chromulina*, but not *Ochromonas*) [3,9] and Synurophyceae (e.g. *Synura*) [3,47].

For the Synurophyceae, there are also comparisons of the *in vitro*  $K_{0.5}$  of Rubisco and *in vivo* photosynthesis. The form ID Rubiscos of the Synurophyceae have  $K_{0.5}$  values ( $\text{mmol m}^{-3}$ ) *in vitro* of 18.2 (*Mallomonas papulosa*), 28.4 (*Synura petersenii*) and 41.8 (*Synura uvella*) [31]. The photosynthetic  $K_{0.5}(\text{CO}_2)$  are 92.0–440.5  $\text{mol m}^{-3}$  for *M. papillosa* (varying with the buffer used to maintain the pH at 7.0), 40.4–43.7  $\text{mol m}^{-3}$  (varying with pH 6–7) for *S. petersenii* and 44.9–209  $\text{mol m}^{-3}$  for *S. uvella* (varying with pH 6–7) [31]. The *in vivo* photosynthetic  $K_{0.5}$  values can be accommodated by the *in vitro* Rubisco  $K_{0.5}$  with diffusive  $\text{CO}_2$  entry, granted a Rubisco content that gives a  $V_{\text{max}}$  for Rubisco equal to the  $V_{\text{max}}$  for *in vivo* photosynthesis, and allowing for the necessary decrease in  $\text{CO}_2$  concentration along the diffusion pathway from the bulk medium to Rubisco [41]. The Rubisco assays used unpurified

cell extracts [31], so the  $\text{CO}_2$ -saturated Rubisco specific reaction rate cannot be calculated.

### (c) Eustigmatophyceae

Eustigmatophyceae appear to all have a CCM, when cells are grown in culture media in equilibrium with the present atmosphere. The freshwater *Eustigmatos vischeri* and *Vischeria stellata* can take up both  $\text{CO}_2$  and  $\text{HCO}_3^-$  [48–50], while the soil-dwelling *Monodus subterraneus* can only use  $\text{CO}_2$  [48,49]. Most of the data are available for the marine *Nannochloropsis* spp. (*N. gaditana*, *N. oceanica* and *N. salina*) and *Monallatus* sp., where mass spectrometric and other evidence show  $\text{HCO}_3^-$  influx powered by mitochondrial respiration, with significant simultaneous efflux of  $\text{CO}_2$  [48,49,51–58]. Merrett *et al.* [53] showed that  $\text{HCO}_3^-$  influx was inhibited by DIDS (4'4'-diisothiocyanostilbene-2,2'-disulfonic acid), an inhibitor of anion exchangers, and by the absence of  $\text{Cl}^-$ , consistent with the occurrence of a  $\text{HCO}_3^- : \text{Cl}^-$  antiport of unknown stoichiometry. The energized  $\text{HCO}_3^-$  influx is downregulated when cells are grown at high  $\text{CO}_2$  [58], consistent with decreased CCM expression at high  $\text{CO}_2$  when diffusive  $\text{CO}_2$  entry can provide as high a  $\text{CO}_2$  concentration at the active site of Rubisco as does the CCM at lower external  $\text{CO}_2$  concentrations. However, Merrett *et al.* [53] were unable to demonstrate a higher internal than external inorganic C concentration using silicone oil centrifugation, just as Huertas *et al.* [48,49] were unable to do from  $\text{CO}_2$  efflux kinetics just after cessation of illumination. Huertas *et al.* [48] suggest that the intracellular pool occupies a small fraction of the cell volume and/or that *Nannochloropsis* Rubisco had, like red algal Rubiscos, a low  $K_{0.5}$  for  $\text{CO}_2$ . This latter suggestion was verified by Tchernov *et al.* [30], who found a  $K_{0.5}$  for  $\text{CO}_2$  of extracted *Nannochloropsis* sp. Rubisco of 7–10  $\text{mol m}^{-3}$ , the lowest values in table 2. This notwithstanding, the need for a CCM exists in *Nannochloropsis* due to a very low  $\text{CO}_2 : \text{O}_2$  selectivity of 27 [30] and incomplete suppression of Rubisco oxygenase activity *in vivo* [59]. Expressing the photosynthetic  $K_{0.5}$  for  $\text{HCO}_3^-$  in terms of  $\text{CO}_2$  gives  $K_{0.5}$  values of 0.27  $\text{mmol m}^{-3}$  for *Monallantus* sp. and 0.63  $\text{mmol m}^{-3}$  for *Nannochloropsis gaditana* [52], i.e. an order of magnitude higher affinity than for the Rubisco of *Nannochloropsis* sp. [30], indicating the involvement of a CCM. Furthermore, the occurrence of a net  $\text{CO}_2$  efflux in the light with all inorganic C entering as  $\text{HCO}_3^-$  and high affinities for inorganic C, a close approximation to inorganic C saturation at seawater inorganic C concentration, a low (zero)

and O<sub>2</sub>-insensitive CO<sub>2</sub> compensation point, a high ability to photosynthesize at high pH and no inhibition of photosynthesis by 21 kPa O<sub>2</sub> relative to 2 kPa O<sub>2</sub> means that there has to be a higher internal than external CO<sub>2</sub> concentration. Pyrenoids are apparently universal in the Eustigmatophyceae (but see [60]), in parallel with the occurrence of CCMs [61,62].

The genomic data show probable HCO<sub>3</sub><sup>-</sup> transporters of the anion exchanger family at the plasmalemma (Nga00165.01) and the chloroplast envelope (Nga06584) of *Nannochloropsis gaditana* CCMP526 [21]; see [www.nannochloropsis.org/gene/Naga\\_10007g30](http://www.nannochloropsis.org/gene/Naga_10007g30) and [www.nannochloropsis.org/gene/Naga\\_10007g124](http://www.nannochloropsis.org/gene/Naga_10007g124). The *Nannochloropsis oceanica* CCMP1779 genome has two genes that resemble the LCIA 1595 in *Chlamydomonas* [63,64]: one is a chloroplast envelope carrier protein (CCP), which is induced by low CO<sub>2</sub> (CCMP1779\_7325-mRNA-1); the other is the LCIA protein (CCMP1779\_6536-mRNA-1), which belongs to the formate/nitrite transporter family, has an unknown location and is also induced under low CO<sub>2</sub> [22]. Using the same strain of *N. oceanica*, Poliner *et al.* [65] found two SL4 HCO<sub>3</sub><sup>-</sup> transporters. The SLC4 family from metazoans catalyses a variety of HCO<sub>3</sub><sup>-</sup> transport processes, i.e. 1 HCO<sub>3</sub><sup>-</sup> : 1 Cl<sup>-</sup> exchanger, 1 HCO<sub>3</sub><sup>-</sup> : 1 Na<sup>+</sup> cotransporter, 2 HCO<sub>3</sub><sup>-</sup> : 1 Na<sup>+</sup> cotransporter, 3 HCO<sub>3</sub><sup>-</sup> : 1 Na<sup>+</sup> cotransporter and a (2 HCO<sub>3</sub><sup>-</sup> + 1 Na<sup>+</sup>) : 1 Cl<sup>-</sup> exchanger [66]. Of these, only the 1 HCO<sub>3</sub><sup>-</sup> : 1 Na<sup>+</sup> cotransporter could lead to inorganic carbon accumulation in the cytosol, granted the probable gradients of Na<sup>+</sup> and Cl<sup>-</sup> across the plasmalemma [67,68]. SLC4 HCO<sub>3</sub><sup>-</sup> transporters are also known from the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* and the brown alga *Ectocarpus siliculosus* [65]. The occurrence of SL4 HCO<sub>3</sub><sup>-</sup> transporters is not diagnostic of CCMs since they are found in the C<sub>3</sub> terrestrial flowering plant *Arabidopsis thaliana* [65] and metazoans [66]. Li *et al.* [69] found two low CO<sub>2</sub>-induced putative formate/nitrite transporters in the plastid envelope of *N. oceanica* IMET1.

#### (d) Phaeophyceae

The largest ochristan algae occur in the Phaeophyceae [16]. The final pH and corresponding equilibrium CO<sub>2</sub> concentration achieved in pH drift experiments with Phaeophyceae show HCO<sub>3</sub><sup>-</sup> use or, less likely, energized accumulation of CO<sub>2</sub> in almost all cases [70–78], with the proviso that the final pH may be restricted by pH *per se* rather than by limitations on the removal of inorganic C from seawater [41,79]. Similar conclusions arise from the meta-analysis by Stepien [80] of δ<sup>13</sup>C natural abundance of organic C of Phaeophyceae collected from their natural habitats, with the proviso that the <sup>13</sup>CO<sub>2</sub> : <sup>12</sup>CO<sub>2</sub> discrimination of the isolated Rubisco of the only ochristan tested, the diatom *Skeletonema costatum* [81], differs from that assumed by Stepien [80] and the authors that she cites.

Of the intertidal and subtidal Phaeophyceae from the northeast Atlantic examined by Johnston & Raven [82–84], Surif & Raven [85,86] and Johnston [32], the intertidal species (Fucales) show high affinities for inorganic C, a close approximation to inorganic C saturation at seawater inorganic C concentration, a low (zero) and O<sub>2</sub>-insensitive CO<sub>2</sub> compensation concentration, a high ability to photosynthesize at high pH and no inhibition of photosynthesis by 21 kPa O<sub>2</sub> relative to 2 kPa O<sub>2</sub>. By contrast, the subtidal Laminariales have a lower affinity for inorganic C, lack of inorganic C saturation at seawater inorganic C concentration, a higher and

O<sub>2</sub>-sensitive CO<sub>2</sub> compensation concentration, a smaller ability to photosynthesize at high pH and some inhibition of photosynthesis by 21 kPa O<sub>2</sub> relative to 2 kPa O<sub>2</sub>. These characteristics indicate the occurrence of a CCM in all species; the CCM of the Laminariales appears to be less developed in terms of inorganic C affinity and O<sub>2</sub> insensitivity of photosynthesis than that of the Fucales. The work of Johnston & Raven [82–84], Surif & Raven [85,86] and Johnston [32] involved experiments under submersed and under emersed conditions with (where identical treatments were used) closely similar results, although the external availability of both HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> in submersed experiments and external availability of only CO<sub>2</sub> in emersed experiments leaves unanswered questions about the mechanism of inorganic C entry to cells.

Why, in ecological and evolutionary terms, is there this difference between intertidal and subtidal brown algae in the northeast Atlantic? We need to look at other possible determinants of zonation, such as tolerance of desiccation, especially for high-intertidal algae, which can be emersed for long periods during neap tides [16]. One would expect that desiccation tolerance would be important for high-intertidal algae, especially when emersed for long periods at spring tides. Surif & Raven [86] suggest that a high affinity for inorganic C of intertidal brown algae in the NE Atlantic increases the photosynthetic C gain per emersion period. However, there are several intertidal (some very high intertidal) red algae lacking CCMs [41], e.g. *Bostrychia arbuscula* (formerly *Stictosiphonia arbuscula*) in New Zealand in the high-intertidal zone occupied by *Pelvetia canaliculata*, a furoid with a CCM, on NE Atlantic coasts [16,87].

The extent of suppression of Rubisco oxygenase and hence of photorespiration by CCMs of Phaeophyceae has been investigated by Surif & Raven [86] using a comparison of photosynthetic rates as a function of CO<sub>2</sub> from 50 to 950 μmol mol<sup>-1</sup> total gas with O<sub>2</sub> at 20 or 210 mmol mol<sup>-1</sup> total gas. The photosynthetic rates of the five intertidal species of the Fucales (Fucales) were unaffected by the different O<sub>2</sub> levels [84] while those of the normally submerged Laminariales and the normally submerged member of the Cystoseiraceae (Fucales) were lower in 210 than in 20 mmol mol<sup>-1</sup> total gas at all the CO<sub>2</sub> levels tested [86]. The O<sub>2</sub> inhibition of the normally submerged algae differs from that of typical C<sub>3</sub> physiology plants by the absence of CO<sub>2</sub>-O<sub>2</sub> competition.

Other data on O<sub>2</sub> inhibition of photosynthesis in the Phaeophyceae [88–92] show increased glycine and serine production (as markers for the photorespiratory carbon oxidation cycle) as a fraction of total inorganic C assimilated with increasing O<sub>2</sub> [90]. The presence of the photorespiratory carbon oxidation cycle in the Phaeophyceae has been shown by enzyme assays [93,94].

Much of the information on other members of the Phaeophyceae show characteristics more like those of normally submerged algae than the intertidal algae studied by Johnston & Raven [82–84], Surif & Raven [85,86] and Johnston [32]. Examples of the inorganic C affinity and O<sub>2</sub> sensitivity of photosynthesis by other members of the Phaeophyceae are provided by Black *et al.* [88], Downton *et al.* [89], Burris [90], Dromgoole [91,92] and Raven *et al.* [71–73]: see review by Raven & Hurd [95].

The general assumption is that the CCM is based on the direct or indirect use of external HCO<sub>3</sub><sup>-</sup> rather than on active influx of CO<sub>2</sub>. HCO<sub>3</sub><sup>-</sup> use could involve direct HCO<sub>3</sub><sup>-</sup> transport using an anion transport protein in the



plasmalemma that is usually detected in macroalgae by inhibition of photosynthesis by DIDS and/or SITS (4'-acetamido-4'-isothiocyanostilbene-2,2-disulfonic acid). Such transport is known for the gametophyte, but not for the sporophyte phase of the kelp *Undaria pinnatifida* [96] and for the sporophyte phase (gametophyte not examined) of the giant kelp *Macrocystis pyrifera* over a wide range of pH values [28]. No inhibition of photosynthesis by DIDS was found for the members of the Desmarestiales, Fucales, Laminariales, Sphacelariales or Scytosiphonales studied by Larsson & Axelsson [97] or Zou & Gao [74]. Evidence of  $\text{HCO}_3^-$  entry in *E. siliculosus* (Ectocarpales) comes from experiments at an external pH of 9.5, where  $\text{CO}_2$  is less than  $0.23 \text{ mmol m}^{-3}$  [98]. In these experiments, the photosynthetic rate at pH 9.5 was still 30% of that at pH 7.9 and was insensitive to buffering of the medium with  $50 \text{ mol m}^{-3}$  of either cationic or anionic pH buffers, or to inhibition of external carbonic anhydrase, thus ruling out the mechanism of  $\text{HCO}_3^-$  use discussed in the two following paragraphs [98].  $\text{HCO}_3^-$  entry could account for part of the photosynthesis of *E. siliculosus* at an external pH of 7.9–8.2 ([98]; cf. [99]). At an external pH of 8 in photosynthesis-saturating red light, *E. siliculosus* accumulates  $4\text{--}5 \text{ mol m}^{-3}$  inorganic C, i.e. about twice the external concentration; this internal pool is decreased to about  $2 \text{ mol m}^{-3}$  when blue light is added, with photosynthetic consumption of the released inorganic C [100].

The alternative mechanism is based on localized acidification of the surface of the organism using energized  $\text{H}^+$  efflux, although the diffusion boundary layer of photosynthesizing cells is at a higher pH than the bulk medium [101]. The acid zones have an equilibrium  $\text{CO}_2\text{:HCO}_3^-$  ratio that increases by an order of magnitude with each pH unit decrease, and there is also an order of magnitude increase in the rate of uncatalysed  $\text{HCO}_3^-$  conversion to  $\text{CO}_2$  [101]. The higher concentration of  $\text{CO}_2$  at the cell surface has two fates; one is to leak back to the bulk phase, the other is a biologically useful diffusion into the cell to Rubisco. This mechanism is commonly supplemented by the presence of extracellular carbonic anhydrase [102]. The generation of surface acid areas to facilitate  $\text{CO}_2$  diffusion towards Rubisco is well characterized in freshwater Charales and some submerged freshwater flowering plants, where each acid zone has an area of more than  $1 \text{ mm}^2$ . Price & Badger [103] showed that this mechanism of  $\text{HCO}_3^-$  use is inhibited by higher concentrations ( $20\text{--}50 \text{ mol m}^{-3}$ ) of pH buffers of an appropriate  $\text{pK}_a$ . Such attempts that have been made to identify acid zones on the surface of the Phaeophyceae during photosynthesis have not identified areas in the square millimetres [95], although the techniques used were not suited to identifying smaller acid areas. Transient surface acidification occurring with blue light added to photosynthesis-saturating red light in a range of brown algae [104] shows that surface acidification can occur during photosynthesis in the Phaeophyceae.

In the absence of direct measurements of sustained localized surface acidification, the occurrence of a CCM of the kind proposed by Walker *et al.* [101] has been suggested (see discussion above) on the basis of inhibition of photosynthesis by pH buffers and by inhibitors of extracellular carbonic anhydrases. Sometimes the absence of inhibition by the  $\text{HCO}_3^-$  transporter inhibitors DIDS and SITS has also been used as negative evidence for acid zone-dependent CCMs. Support for a Walker *et al.* style [101] CCM by the use of purported inhibitors of  $\text{H}^+$  efflux catalysed by plasmalemma

$\text{H}^+$ -ATPase is equivocal because the  $\text{H}^+$  gradient generated could also be used to indirectly energize a  $\text{Na}^+\text{--HCO}_3^-$  symport influx of  $\text{HCO}_3^-$ . With these provisos, the mechanism that occurs in the Laminariales other than gametophytes of *U. pinnatifida* and sporophytes of *M. pyrifera* is consistent with a mechanism based on that described by Walker *et al.* [101] for *Chara* ([105–107]; see also [108,109]), as is some evidence for this mechanism in a member of the Scytosiphonales [74], but not for a member of the Fucales [77]).

Despite the occurrence of CCMs, most brown algae lack pyrenoids although these structures have evolved independently in several clades of the Phaeophyceae [9,28,78,110,111].

The genome of *Ectocarpus siliculosus* contains a putative  $\text{Na}^+\text{--HCO}_3^-$  transporter targeted to the chloroplast, and a putative  $\text{Cl}^-\text{--HCO}_3^-$  transporter with no clear targeting [112,113]. Nakajima *et al.* [11] and Poliner *et al.* [65] found two SLC4  $\text{HCO}_3^-$  transporters in the genome of *E. siliculosus*; other Ochrophyta have two (in the eustigmatophycean *N. oceanica* and the centric diatom *T. pseudonana*) and four (in the pennate diatom *P. tricornutum*) SLC4 genes. The *P. tricornutum* PtSLC4-2 is targeted to the plasmalemma, is inhibited by the anion transporter inhibitor DIDS and is  $\text{Na}^+$ -dependent. Ye *et al.* [114] report the genome sequence of the kelp (Laminariales) *Saccharina japonica*; the analysis by Bi & Zhou [115], however, does not mention  $\text{HCO}_3^-$  transporters or  $\text{H}^+$ -ATPases, but does show a range of carbonic anhydrases. Genomic data indicate the occurrence of many of the enzymes of the photorespiratory carbon oxidation cycle [112].

### (e) Raphidophyceae and Tribophyceae

Little is known of the inorganic C acquisition by the Raphidophyceae, other than Rubisco kinetics [33,116]. The *in vitro*  $\text{K}_{0.5}$  for  $\text{CO}_2$  of Rubisco in the marine *Olisthodiscus luteus* is  $45 \text{ mmol m}^{-3}$  at  $23^\circ\text{C}$  (table 2); for the marine *Heterosigma carterae*, the  $\text{K}_{0.5}$  for inorganic C dependence of the rate of photosynthesis *in vivo*, expressed in terms of  $\text{CO}_2$ , is  $3 \text{ mmol m}^{-3}$  at  $16^\circ\text{C}$  [117]. This difference suggests that a CCM is operative in the algae of this class. The pH drift work of Nimer *et al.* [118] indicated low photosynthetic rates for the marine *Heterosigma akashiwo*; little can be deduced from these data about the mode of inorganic C acquisition. Pyrenoids seem to be universal in the Raphidophyceae [119]. For the Tribophyceae (i.e. Xanthophyceae), the only data are those of Beardall and Entwisle [120] on the terrestrial–freshwater *Botrydiopsis intercedens*. In this species, internal inorganic C accumulation occurs to a greater extent than it would for diffusive  $\text{CO}_2$  entry, in accord with the pH gradient; this is suggestive of the operation of a CCM. Several members of the Tribophyceae have pyrenoids [9] though there seem to be no data on *B. intercedens*. There are no data on inorganic C acquisition by the sister clade to the diatoms, the Bolidiophyceae, now known to have the Parmales as the silicified cyst phase [121].

### (f) Pyrenoids and $\text{CO}_2$ concentrating mechanisms

As indicated above for individual taxa, pyrenoids seem to be essential features of some CCMs based on active transport across membranes [9,113]. However, pyrenoids occur in some species of the Chrysophyceae and Synurophyceae, classes that uniformly lack CCMs, while the Phaeophyceae, apparently with CCMs in all taxa, generally lack pyrenoids, with several independent origins of pyrenoids. In the Eustigmatophyceae, all members investigated have CCMs and

pyrenoids. In the Synchronophyceae, a condensed pyrenoid is often present in the chloroplast stroma [8], although there is no evidence on the occurrence of CCMs in this class. This is also the case for the Pelagophyceae, in which *Aureaumbra* (at least) has pyrenoids [122].

## 4. Inorganic C assimilation in Ochrophyta other than diatoms

### (a) Introduction

All photosynthetic, eukaryotic C assimilation pathways have the photosynthetic carbon reduction cycle (PCRC, or Calvin–Benson cycle), with Rubisco as the carboxylase, at their core. Raven *et al.* [25] discussed alternative inorganic C assimilation pathways found in some autotrophic Archaea and Bacteria, and energetic, inorganic C affinity and O<sub>2</sub> damage as reasons why many of them are not appropriate for functioning in the present atmosphere. In some organisms, the PCRC is downstream of diffusive entry, in others it is downstream of a ‘biophysical’ CCM based on active transport of HCO<sub>3</sub><sup>-</sup>, CO<sub>2</sub> or H<sup>+</sup> to produce C<sub>3</sub> photosynthetic biochemistry. In some oxygenic photosynthetic organisms, the PCRC is downstream of a C<sub>3</sub>–C<sub>4</sub> cycle in C<sub>4</sub> photosynthesis; this is a ‘biochemical’ CCM, although it occurs downstream of a ‘biophysical’ CCM in some aquatic flowering plants. In C<sub>4</sub> photosynthesis, there is close temporal coupling of the C<sub>3</sub>–C<sub>4</sub> and PCRC cycles with small pool sizes of the C<sub>4</sub> and C<sub>3</sub> intermediates. Another upstream C<sub>3</sub>–C<sub>4</sub> cycle contributes to crassulacean acid metabolism (CAM); here there is a temporal lag of about 12 h between the scotophase acidification with CO<sub>2</sub> fixation into malate, which is stored as malic acid in vacuoles, and deacidification in the photophase, where CO<sub>2</sub> is released by decarboxylation of malic acid, and refixed by Rubisco and the PCRC, with the C<sub>3</sub> moiety from malate decarboxylation stored until the next scotophase as mono- or polysaccharide.

The biochemistry of autotrophic CO<sub>2</sub> assimilation was determined in pre-molecular biology by two main methods. One was short-time (seconds) exposure of the organism to <sup>14</sup>CO<sub>2</sub> (terrestrial organisms) or <sup>14</sup>C inorganic C (aquatic organisms) in the light, followed by rapidly killing the organism and quantification of the water- or ethanol-soluble organic compounds labelled with <sup>14</sup>C. C<sub>3</sub> biochemistry is characterized by 3-phosphoglycerate (PGA) as the initial labelled compound, followed by PCRC sugar phosphates and then compounds derived from the PCRC; labelling of C<sub>4</sub> dicarboxylic acids in anaplerotic processes is only a few percent of that through the PCRC. C<sub>4</sub> biochemistry has C<sub>4</sub> dicarboxylic acids as the initial labelled products, followed by PGA and other PCRC compounds; a few seconds labelling (pulse) followed by a change back to unlabelled inorganic C (chase) shows a decrease in label in PGA and an increase in label in C<sub>4</sub> dicarboxylic acids. This was the method used in the 1950s and 1960s to establish the C<sub>3</sub> pathway of autotrophic inorganic C assimilation in green microalgae (*Chlorella*, *Scenedesmus*) and the terrestrial flowering plant *Hordeum*, and the C<sub>4</sub> pathway in the terrestrial flowering plants *Saccharum* and *Zea*. The other, much less decisive, method is determination of the activity of carboxylase enzymes in cell extracts; a high Rubisco : PEPC (phosphoenolpyruvate carboxylase-oxygenase) activity ratio has been taken to indicate C<sub>3</sub> biochemistry, and a low ratio (less than 1) is suggestive of C<sub>4</sub> biochemistry.

### (b) Phaeophyceae

Both the kinetics of labelling of organic compounds after addition of <sup>14</sup>C inorganic C and the *in vitro* activity of carboxylases methods have been applied to the Phaeophyceae [32,123–128]. The kinetics of the <sup>14</sup>C-inorganic C labelling method shows C<sub>3</sub> biochemistry in a range of brown algae [32,123,127–129]. In the brown algae examined by Küppers & Kremer [130], Kremer [129] and Hillrichs & Schmid [127] there is significant labelling of C<sub>4</sub> acids (aspartate, malate) in the light, and the time course of the label position within aspartate shows that the PEP comes from photosynthetic 3-PGA, with slower incorporation of <sup>14</sup>C-inorganic C into the β-carboxyl of aspartate by phosphoenolpyruvate carboxylase (PEPCK) or, more likely, PEPC. The ratio of <sup>14</sup>C-inorganic C labelling of organic C (initially mainly into aspartate and malate) by carboxylation of PEP to that carried out by Rubisco is lowest in mature tissue and highest in growing tissue, consistent with an anaplerotic role of PEP carboxylation, although the rates may be higher than the anaplerotic requirement [129,130]. <sup>14</sup>C-inorganic C labelling of organic C (mainly aspartate and malate) occurs in the dark at a rate higher than that of green and red marine macroalgae and is also probably higher than the anaplerotic requirement [129]. As indicated below, in some fucoids, a part of this dark <sup>14</sup>C-inorganic C assimilation can be attributed to low amplitude CAM.

Possibly related to the labelling of C<sub>4</sub> acids mentioned above is the finding of a ‘buffer system’ taking up (at high pH in the dark) and releasing (at normal seawater pH in the light) inorganic C, as indicated by stimulation of O<sub>2</sub> production in the light in North Atlantic intertidal members of the Fucales (species of *Ascophyllum*, *Fucus* and *Pelvetia*; all Fucales) [131,132]. This ‘buffer system’ is associated with H<sup>+</sup> exchange between seawater and the algae, is not found in other subtidal brown algae examined, i.e. *Halidrys*, Fucales (Cystosieraceae), *Desmarestia* (Desmarestiales) and *Laminaria* (Laminariales), and is paralleled by a particular spatial organization of organelles in the outer cell layer (meristoderm) of the thallus [131,132]. A more widespread phenomenon among brown algae is the stimulation of photosynthesis by additional blue light to already saturating red light [133,134], which involves release in blue light of at least some of the inorganic C from an intracellular pool accumulated in red light [100]. The blue light effect was not found in the only diatom examined, *P. tricorutum* [133]. Possibly related to the two preceding sets of data, but probably not, is the interesting finding in the work of Johnston [32] on <sup>14</sup>C inorganic C pulse-chase incorporation in the North Atlantic intertidal *Ascophyllum nodosum* of a continued increase in label incorporation in organic compounds in the chase period, in algae collected in July 1998 but not in January 1988. After a 5 s pulse in July 1988, the increase in label of organic C seems to be saturated at the longest time period tested (300 s) at about eight times the organic C label at the end of the pulse period. This requires a very substantial accumulation of an acid labile pool of either inorganic C or of an acid-labile organic C compound in the 5 s pulse, with incorporation into a range of acid-stable compounds in the chase period. Kawamitsu and Boyer [135] used the North Atlantic intertidal *Fucus vesiculosus* and showed that, after exposure to light in seawater, photosynthetic O<sub>2</sub> production continued for a decreasing rate over 2 h in CO<sub>2</sub> free air. A small fraction of the C store on which this O<sub>2</sub> production was based was inorganic C; most of it was organic, presumably (an

organic acid(s). Unlike CAM, and the work of Axelsson *et al.* [131,132], this C store was not filled during the scotophase [135], although there is evidence of minor CAM inorganic C assimilation in several furoid brown algae including *F. vesiculosus* [136]. Axelsson *et al.* [131,132] comment that their 'photosynthetic buffer' may be related to the very low CAM activity found in the species showing the 'photosynthetic buffer'.

The enzyme analyses showed significant activity of PEPCK, but higher activities of Rubisco [32,123,126]. PEPCK activity could not be detected in these investigations, including that of Busch & Schmid [126] on *E. siliculosus*, although genomic evidence indicates that PEPCK occurs in this alga [112].

The most recent tool for distinguishing between C<sub>3</sub> and C<sub>4</sub> (and CAM) photosynthetic biochemistry is genomics and transcriptomics. However, the presence of genes encoding enzymes used in C<sub>4</sub> (and CAM) photosynthesis such as PEPCK, PEPCK, PPKK (phosphate pyruvate dikinase, sometimes considered for diagnostic C<sub>4</sub> photosynthesis), MDH (malate dehydrogenase), NADP-ME (NADP malic enzyme) and NAD-ME (NAD malic enzyme), is not of itself sufficient to show that C<sub>4</sub> photosynthesis occurs. One reason is that the enzymes have other functions and occur in photosynthetic organisms known to use C<sub>3</sub> biochemistry [137,138]. Another is that the enzyme might not be expressed in an intracellular location compatible with C<sub>4</sub> photosynthesis [139]. Bi & Zhou [115] have produced a generalized diagram (their fig. 1) including C<sub>4</sub> photosynthesis and list genes [114] compatible with the PEPCK C<sub>4</sub> mechanism (granted appropriate intracellular localization of the enzymes), but this is not evidence demonstrating that C<sub>4</sub> photosynthesis occurs. The conclusion of Gravot *et al.* [112] is that there is not clear evidence from genome analysis as to the occurrence of C<sub>4</sub> photosynthesis in the model brown alga *E. siliculosus*.

The other inorganic C assimilation pathway found in the Phaeophyceae is very low amplitude CAM in some members of the Fucales, but not in other orders of the brown algae [112,136].

### (c) Raphidophyceae and Eustigmatophyceae

For other photosynthetic Ochrophyta, Descolas-Gros & Oriol [140] found activity of PEPCK but not PEPCK in *H. akashiwo* (Raphidophyceae). However, PEPCK was found in the proteome of another raphidophycean, *Aureococcus anophagefferens* [141].

Genomic data on *N. gaditana* (Eustigmatophyceae) shows PEPCK, MDH (NAD(P) malic dehydrogenase) and PPKK in the cytosol, and NAD(P)-ME in the chloroplast, that could be part of C<sub>4</sub> photosynthetic biochemistry, as well as pyruvate carboxylase (PC) in the mitochondria (fig. 6 of [21]).

Genomic data on *N. oceanica* CCMP1779 (Eustigmatophyceae) show the presence of PEPCK, MDH, PPKK and NAD(P)-ME, but the intracellular location is not indicated (table S10 of [22]). Vieler *et al.* [22] (their table S10) show that expression of probable inorganic C transporters is increased at low CO<sub>2</sub> concentrations, but make no comment about the effects of CO<sub>2</sub> on expression of the enzymes that could be involved in C<sub>4</sub> photosynthesis. Li *et al.* [69] used transcriptomic data on *N. oceanica* IMET1 to predict the location of enzymes that could be part of a C<sub>4</sub> photosynthesis and found that enzyme location is not entirely as required for an effective C<sub>4</sub> pathway.

**Table 3.** Summary of mechanisms of inorganic carbon acquisition and assimilation among classes in the Ochrophyta. No data are available for the Aureanophyceae, Bolidophyceae, Chrysochromophyceae, Dictyochophyceae, Pelagophyceae, Phaeothamniophyceae, Pinguicophyceae, Schizodactylophyceae and Synchronophyceae. See the main text for details.

class	Rubisco kinetics	diffusive CO <sub>2</sub> entry	CCM	C <sub>3</sub> biochemistry	C <sub>4</sub> biochemistry	CAM	phagotrophy
Bacillariophyceae	yes	no	yes	yes	C <sub>3</sub> -C <sub>4</sub> in some?	no	no
Chrysophyceae	no data	yes	no	assumed C <sub>3</sub>	no data	unlikely	no
Eustigmatophyceae	yes	no	yes	assumed C <sub>3</sub>	?	unlikely	no
Phaeophyceae	yes	no	yes	yes	some production of C <sub>4</sub> acids in the light	low amplitude in some species	low amplitude in some species
Raphidophyceae	yes	probably not	probably yes	no data	no data	unlikely	no
Synurophyceae	yes	yes	no	no data	no data	unlikely	no
Tribophyceae	no data	no	yes	no data	no data	unlikely	no



## 5. Conclusion

We only have a significant body of information on inorganic C acquisition and assimilation for four non-diatom classes of Ochrophyta, i.e. the Chrysophyceae, Eustigmatophyceae, Phaeophyceae and Synurophyceae, with some information on the Raphidophyceae and Tribophyceae (table 3). Diffusive CO<sub>2</sub> entry from the bulk medium to Rubisco occurs in the Chrysophyceae and Synurophyceae, while the Eustigmatophyceae and Phaeophyceae have CCMs involving influx of HCO<sub>3</sub><sup>-</sup> and/or CO<sub>2</sub> (table 3).

The Phaeophyceae is the only class for which there is biochemical evidence on the pathway of inorganic C assimilation, showing that there is predominantly C<sub>3</sub> biochemistry, but with occasional elements of C<sub>4</sub> biochemistry and low-amplitude CAM in some members of the Fucales.

The energization of CCMs involves active influx of HCO<sub>3</sub><sup>-</sup> or active efflux of H<sup>+</sup> at the plasmalemma, with little or no role for C<sub>4</sub> biochemistry, and no net inorganic C entry in the dark in the few brown algae with low amplitude CAM. The distribution of pyrenoids does not completely parallel that of CCMs based on active transport across membranes.

Molecular genetic investigation has not provided definitive evidence as to the occurrence of C<sub>4</sub> photosynthesis and has indicated possible membrane transporters involved in CCMs. Some members of the Fucaceae, and *Ectocarpus*, have

incompletely explained inorganic C reservoirs, as inorganic C and/or as (presumably) carboxylate C that can be readily converted to inorganic C.

Returning to the topic of the other papers in this thematic issue, i.e. C metabolism in the Bacillariophyceae, the other classes of Ochrophyta for which information is available show a greater diversity of mechanisms of inorganic C acquisition and biochemistry of autotrophic CO<sub>2</sub> assimilation. The occurrence of CCMs is less widespread among other ochrophytes than in diatoms, as is the correlation between the occurrence of CCMs and the presence of pyrenoids. While C<sub>4</sub> or C<sub>4</sub>-like photosynthetic metabolism is a possibility in both diatoms and other ochrophytes, no diatom is known to have the low-amplitude CAM found in some fuclean brown algae.

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## Correction



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# Correction to 'Acquisition and metabolism of carbon in the Ochrophyta other than diatoms'

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There are errors in column 8 (Phagotrophy) of table 3. The errors concerned the distribution of phagotrophy among photosynthetic stramenopiles; these errors do not conflict with table 1 or the rest of the paper. The corrected form of table 3 is presented here. The authors apologize for any inconvenience.

**Table 3.** Summary of mechanisms of inorganic carbon acquisition and assimilation among classes in the Ochrophyta. No data are available for the Aureanophyceae, Bolidophyceae, Chrysomeridophyceae, Dictyochophyceae, Pelagophyceae, Phaeothamniophyceae, Pinguiphyceae, Schizocladiophyceae and Synchronophyceae. See the main text for details.

class	Rubisco kinetics	diffusive CO <sub>2</sub> entry	CCM	C <sub>3</sub> biochemistry	C <sub>4</sub> biochemistry	CAM	phagotrophy
Bacillariophyceae	yes	no	yes	yes	C <sub>3</sub> –C <sub>4</sub> in some?	no	no
Chrysophyceae	no data	yes	no	assumed C <sub>3</sub>	no data	unlikely	yes
Eustigmatophyceae	yes	no	yes	assumed C <sub>3</sub>	?	unlikely	no
Phaeophyceae	yes	no	yes	yes	some production of C <sub>4</sub> acids in the light	low amplitude in some species	no
Raphidophyceae	yes	probably not	probably yes	no data	no data	unlikely	yes
Synurophyceae	yes	yes	no	no data	no data	unlikely	no
Tribophyceae	no data	No	yes	no data	no data	unlikely	no