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Inorganic carbon concentrating mechanisms in free-living and symbiotic dinoflagellates and chromerids

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Abstract

Photosynthetic dinoflagellates are ecologically and biogeochemically important in marine and freshwater environments. However surprisingly little is known of how this group acquires inorganic carbon or how these diverse processes evolved. Consequently, how CO_2 availability ultimately influences the success of dinoflagellates over space and time remains poorly resolved compared to other microalgal groups. Here we review the evidence. Photosynthetic core dinoflagellates have a Form II RuBisCO (replaced by Form IB or Form ID in derived dinoflagellates). The in vitro kinetics of the Form II RuBisCO from dinoflagellates are largely unknown, but dinoflagellates with Form II (and other) RuBisCOs have inorganic carbon concentrating mechanisms (CCMs), as indicated by in vivo internal inorganic C accumulation and affinity for external inorganic C. However, the location of the membrane(s) at which the essential active transport component(s) of the CCM occur(s) is (are) unresolved; isolation and characterisation of functionally competent chloroplasts would help in this respect. Endosymbiotic Symbiodiniaceae (in Foraminifera, Acantharia, Radiolaria, Ciliata, Porifera, Acoela, Cnidaria and Mollusca) obtain inorganic C by transport from seawater through host tissue. In corals this transport apparently provides an inorganic C concentration around the photobiont that obviates the need for photobiont CCM. This is not the case for tridacnid bivalves, medusae, or, possibly, Foraminifera. Overcoming these long-standing knowledge gaps relies on technical advances, e.g. the in vitro kinetics of Form II RuBisCO, that can functionally track the fate of inorganic C forms.

Key Words: Chromerids; Corals; Dinoflagellates; Inorganic carbon concentrating mechanisms; Mixotrophy; Photosythesis; Rubisco; Symbiodiniaceae; Symbiosis; Tridacnids

Abbreviations: AZA: Acetazolamide; CA: Carbonic anhydrase; CCM: CO_2; concentrating mechanism; DBL: Diffusion boundary layer; DBS: Dextran-bound sulphonamide; DIDS: 4′4′-diisothiocyanostilbene-2,2-disulfonic acid; EZA: Ethoxyzolamide; $K_{0.5}$: Substrate concentration giving half the substrate-saturated rate of an enzyme or transporter; MIMS: Membrane inlet mass spectrometry; rETR: Relative electron transport rate through photosystem II; RuBisCO: Ribulose-1,5-bisphosphate carboxylase-oxygenase; SITS:

Disodium-4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulphonate; SNARF: Seminaphtharhodafluor;
TRIS: Tris(hydroxymethyl)aminomethane; VPDB: Vienna Pee-Dee Belemnite

Introduction

The Class Dinophyceae, with chromerids, ciliates, and apicomplexans, make up the Alveolata, which is in turn a members of the TSAR (Telonemia, Stramenopila, Aleveolats, Rhizaria) Supergroup (Strassert et al. 2018, 2019, Burki et al. 2020). The Dinophyceae are photolithotrophic (Janouškovec et al. 2017), mixotrophic (photophagotrophic: Jeong et al. 2010, Stoecker et al. 2017, Janouškovec et al. 2019) or chemo-organotrophic (Jeong et al. 2010, Janouškovec et al. 2017), including parasitic (Coats 1999) organisms. Dinophyceae are common as free-living organisms in freshwaters, and free-living, or as endosymbionts (= inhabitants) of non-photosynthetic organisms, in the ocean.

Basal (peridinin- and Form II RuBisCO-containing, except when secondarily non-photosynthetic) dinoflagellates became photosynthetic through secondary endosymbiosis of a red alga, a member of the Archaeplastida whose chloroplasts are products of the primary endosymbiosis of a β-cyanobacterum with subsequent genetic integration (Janouškovec et al. 2017, Janouškovec et al. 2019). In the red algae, the Form ID RuBisCO (ribulose bisphosphate carboxylase-oxygenase) inherited with the plastids from the ancestral cyanobacterium was replaced with a Form II RuBisCO from a proteobacterium through horizontal gene transfer or, possibly, endosymbiotic gene transfer (Imanian et al. 2012, Dorrell and House 2015, Dorrell et al. 2016a,b, 2019). These basal dinoflagellates, with three chloroplast envelope membranes, were thought to be the only photosynthetic eukaryotic organisms with Form II Rubisco until their sister group, the marine symbiotic Chromerida (*Chromera velia* and *Vitrella brassiciformis*) with four chloroplast envelope membranes, were discovered (Dodge 1968, Moore et al. 2008, Obornik et al. 2012, Venuleo et al. 2018, Janouškovec et al. 2019). Form II Rubiscos from Bacteria, for which kinetics have been determined, have low CO_2 affinity and low CO_2-O_2 selectivity, making a CO_2 concentrating mechanism (CCM) essential for photosynthesis in air-equilibrated fresh water or seawater (Whitney and Andrews 1998). As discussed below, the low in vitro half-life of dinoflagellate

RuBisCO means that only the CO_2-O_2 selectivity has been determined (Whitney and Andrews 1998), Despite the essential roles of RuBisCO – and therefore CCMs – in sustaining these ecologically and biogeochemically important algae (Mayfield et al. 2014, Murray et al. 2016), we in fact know very little of how these cellular constituents function, and therefore govern ecological dynamics. This paper therefore reviews and integrates the current knowledge of the eukaryote Form II Rubiscos, and the nature of the CCM for free-living and symbiotic photosynthetic alveolates.

Dinoflagellate RuBisCO diversity beyond Form II

In some dinoflagellates the peridinin- and Form II RuBisCO- containing plastids have been replaced in tertiary endosymbiosis (Schnepf and Elbrächter 1999, Dorell and Howe 2015, Janouškovec et al. 2017, Matsuo and Inagaki 2018). One group involves core chlorophytes (mainly *Lepidodinium*) with chlorophyll b and Form IB RuBisCO (Watanabe et al. 1990, Minge et al. 2010, Zapata et al. 2012, Kamikawa et al. 2015, Matsumoto et al. 2015, Jackson et al. 2018), with the core chlorophyte *Pedinomonas* as the closest living relative of the inhabitant that gave rise to the chloroplast. Three other groups involve cryptophytes (*Dinophysis*) with phycobiliproteins and Form ID Rubiscos, haptophytes (*Karenia*, *Karlodinium*) with 19'hexanoyloxyfucoxanthin and Form ID RuBisCO, and diatoms ('dinotoms': *Durinskia*, *Kryptoperidinium*) with fucoxanthin and its derivatives and Form ID Rubisco as the tertiary endosymbionts (Tengs et al. 2000, Janson and Granéli 2003, Bachvaroff et al. 2009, Imanian et al. 2012, Zapata et al. 2012, Hohenberger et al. 2014, Dorrell and Howe 2015, Kretschmann et al. 2018, Yamada et al. 2020). The chlorophyte and haptophyte endosymbionts did not retain their mitochondria and nuclei in their integration to form chloroplasts with four membranes (Dorrell and Howe 2015, Dorell et al. 2015). The diatom endosymbionts did retain their nucleus and mitochondria in genetic integration, with a single membrane round nucleus, mitochondria and the plastids with four surrounding membranes (Imanian et al. 2012, Hehenberger et al. 2014, Dorrell and Howe 2015). There is evidence of the remains of the original (basal dinoflagellate) plastids in dinotoms (Hehenberger et al. 2014).

Some dinoflagellates (*Citharistes, Dinophysis*, *Histoneis*, *Ornithocercus, Sinophysis*) have intracellular cyanobacteria (Gordon et al. 1994, Foster et al. 2006, Escalara et al. 2011,

Qiu et al. 2011). The cyanobacteria have photosynthetic pigments, thylakoids and carboxysomes, and, where investigated (*Histoneis*, *Ornithoceros*), have nitrogenase (Foster et al. 2006) and are β-cyanobacteria (Escalara et al 2011), and so have cyanobacterial Form IB RuBisCO. Nakayama et al. (2019; see also Archibald 2019) found an extracellular symbiotic α-cyanobacterium (so with cyanobacterial Form IA RuBisCO) associated with the pelagic marine heterotrophic dinoflagellate *Ornithocercus magnificus*. Some evidence is consistent with the dinoflagellate 'farming', ingesting and digesting, the cyanobacteria (Archibald 2019, Nakayama et al. 2019).

Finally, *Dinophysis* spp. have (presumably) short-lived kleptoplastids derived from prey algae, including Prasinophyceae (Form IB Rubisco), and, all with Form ID RuBisCO, Cryptophyta, Haptophyta and Ochrophyta (Bacillariophyceae, Dictyochophyceae, Pelagophyceae; Daugbjerg and Henriksen 2001, Park et al. 2010, Qiu et al. 2011, Nishitani et al. 2012, Rial et al. 2012, Hongo et al. 2018). An Antarctic dinofagellate related to *Karenia* and *Karlodinium* has haptophyte kleoplastids (Gast et al. 2007). *Gymnodinium eucaryum* has cryptophyte kleptoplastids (Xia et al. 2013). *Nasuttodinium* gen. nov. is a kleptoplastidic genus of unarmoured dinoflagellates; the five species were previously assigned to the genera *Amphidinium* and *Gymnodinium* (Takano et al. 2014). Chloroplast replacement in dinoflagellates is summarized in Table 1.

In the context of CCMs related to 'Post Form II RuBisCO' photosynthetic capacity in dinoflagellates, the cyanobacterial symbionts have Form IB (cyanobacterial) or Form IA (cyanobacterial) RuBisCOs with relatively low CO_2 affinity and low CO_2:O_2 selectivity, while the Form IB (green algae) and Form ID RuBisCOs of the plastids derived by tertiary endosymbosis or kleptoplasty have higher CO_2 affinity and CO_2:O_2 selectivity (Whitehead et al. 2014, Shih et al. 2016, Bathellier et al. 2018, Flamholz et al. 2019)

Basal dinoflagellate Form II RuBisCO and the need for a CCM

Basal (peridinin-containing) dinoflagellates have a Form II RuBisCO (ribulose bisphosphate carboxylase-oxygenase) encoded in the nucleus (Morse et al. 1995, Palmer 1996, Rowan et al. 1996). The Form II RuBisCOs of dinoflagellates have, like other Form II RuBisCOs, a low CO_2:O_2 selectivity (Whitney and Andrews 1998, Bathellier et al. 2018, Flamholz et al. 2019). The short in vitro half-life of activity of the dinoflagellate Form II RuBisCOs meant that early

research had to develop a novel method to determine the CO_2:O_2 selectivity (Whitney and Andrews 1998) involving 1-^{13}C-labelled-D-ribulose-1,5-bisphosphate as substrate with $^{12}CO_2$ and O_2 for the *Amphidinium carterae* RuBisCO carboxylase-oxygenase in vitro. This method allowed sufficient labelling of the product 3-phosphoglycerate in the short in vivo activity of the enzyme to calculate the carboxylase:oxygenase reaction from the ratio of labelled to unlabelled 3-phosphoglycrate, since the carboxylase activity yields equal numbers of ^{13}C-labelled and unlabelled phosphoglycerate wheeras the oxygenase reaction yields only unlabelled phosphoglycerate. Lilley et al. (2010) used chemiluminescence to assay the activity of Rubisco oxygenase in extracts of *Symbiodinium* sp. with Mn^{2+} rather than Mg^{2+} as the activating divalent cation, and the Mn^{2+} affinity of the oxygenase reaction. Data on Form IB RuBisCO with Mn^{2+} rather than the dominant in vivo Mg^{2+} as the activating cation yields similar oxygenase activity but much lower carboxylase activity (Wildner and Henkel 1979). Subsequent work on flowering plant Form IB RuBisCO, and Form II RuBisCO from the alpha-proteobacterium *Rhodospirillum rubrum*, demonstrated a lower carboxylase:oxygenase ratio with Mn than with Mg (Bloom and Kameritsch 2017).

So far, no methods have been developed to determine other kinetic parameters of the dinoflagellate Form II RuBisCO. However, by analogy with bacterial Form II RuBisCOs, the $K_{0.5}$ CO_2 – the half saturation constant for CO_2 – is likely to be high (Whitney and Andrews 1998, Bathellier et al. 2018, Flamholz et al. 2019). Thus, with such a Rubisco, a CCM is essential for photosynthesis in air-equilibrium seawater or fresh waters (Badger et al. 1998, Raven et al. 2017).

One possibility for a CCM is C_4 photosynthesis, i.e., a biochemical CCM. C_4 photosynthesis involves fixation of external CO_2 (as HCO_3^-) by phosphoenolpyruvate carboxylase in the cytosol to yield the C_4 dicarboxylate oxaloacetate; decarboxylation of the C_4 dicarboxylate producing CO_2 occurs in a compartment containing Rubisco and with limited possibility of CO_2 diffusion back to the medium. The other, C_3 monocarboxylate, product of decarboxylation is returned to the cytosol and, using ATP, reconverted to the phosphoenolpyruvate used by the phosphoenolpyruvate carboxylase. The steady-state CO_2 concentration at the site of Rubisco is higher than that in the medium, increasing the CO_2:O_2 ratio available to Rubisco in the C_3 Calvin-Benson-Bassham cycle.

The distinction between C_3 and C_4 biochemistry in photosynthetic organisms is most clearly achieved using short term ^{14}C-inorganic C labelling with separation and quantitation of the products using (preferably high- or ultra-performance) chromatography. 'Short term' is a labelling time of a few seconds, and so labelling of cells from the Family Symbiodiniaceae (sensu LaJeunesse et al. 2018) cells in hospite, for 3 min as the shortest labelling time, cannot distinguish the biochemical pathway of autotrophic inorganic C assimilation (Schmitz and Kremer 1977). In contrast, 5 s rapid labelling for Symbiodiniaceae that is possible for ex hospite (i.e., isolated from the exhabitant) cells shows that 58% of the ^{14}C labelled organic material is in the C_3 compound 3-phosphoglycerate, which is the first stable product of Rubisco carboxylase (Streamer et al. 1993). Ten percent of the organic label is in the C_4 dicarboxylate aspartate, which is produced by carboxylation by a C_3+C_1 (anaplerotic) carboxylase. This enzyme in basal, peridinin-containing, dinoflagellates can be pyruvate carboxylase in *Amphidinium carterae, Amphidinium operculatum, and Scripsiella trochoidea* (Appleby et al. 1980, Descolas-Gros and Fontugne 1985, Descolas-Gros and Oriol 1992), phosphoenolpyruvate carboxylase in *Prorocentrum micans* (Descolas-Gros and Oriol 1992) and phosphoenolpyruvate carboxykinase in the non-photosynthetic *Cryptothecodinium cohnii* (Descolas-Gros and Oriol 1992). The 5 s ^{14}C-inorganic C labelling does not yield products associated with RuBisCO oxygenase activity (Streamer et al. 1993); this is consistent with the O_2-affinity characteristics of light stimulated $^{18}O_2$ uptake that resembles a water-water cycle (e.g., Mehler-Peroxidase or flavodi-iron O_2 uptake) rather than Rubisco oxygenase (Leggat et al. 1999, Badger et al. 2000). Together these characteristics are in agreement with the occurrence of a CCM, although it should be noted that the experimental ideal of 'instantaneously' replacing ^{12}C-inorganic C with the same concentration of ^{14}C-inorganic C is very difficult to achieve for free-living dinoflagellates, and even more difficult for dinoflagellates as endosymbionts.

The dinoflagellates with tertiary endosymbiotic plastids or kleptoplastids have Form ID, or green algal Form IB RuBisCO, with higher affinities for CO_2 and greater CO_2:O_2 selectivity than the Form II Rubisco of the basal dinoflagellates (Raven et al. 2017, Bathellier et al. 2018, Flamholz et al. 2019). This generally involves the presence of a CCM in present day air-equilibrium waters, but with less obvious requirement with future predicted CO_2 concentrations (e.g., the year 2100; Raven et al. 2017). By contrast, peridinin-containing

dinoflagellates with Form II Rubisco will still need CCMs under predicted 2100 air-equilibrium CO_2 concentrations. This requirement for CCMs might be partly offset in some coastal and estuarine waters, and fresh waters, with above air equilibrium CO_2 concentrations (Raven et al. 2017, 2020), and in ocean anoxic zones (Breitburg et al. 2018, Machu et al. 2009, Penn et al. 2019). Phagomixotrophic dinoflagellates have an internal CO_2 source from metabolism of ingested organic particles, although this additional supply does not replace a CCM when the phagomixotrophs have net assimilation of external inorganic carbon (Raven et al. 2020).

Evidence related to CCMs: intracellular CO_2 concentrations

Berman-Frank and Erez (1996) investigated the intracellular inorganic carbon pool in the basal freshwater dinoflagellate *Peridinium gatunense* by pulse labelling with ^{14}C inorganic C, followed by a wash in ^{14}C-free medium and then measurement of increase in ^{14}C organic carbon (from the intracellular inorganic ^{14}C pool). Using this approach, the intracellular:extracellular inorganic C ratio is 21-78 with external inorganic C in the range 2016-2177 mmol $\cdot$ m^{-3}, internal inorganic C in the range 44 – 170 (mean 118) mol $\cdot$ m^{-3} and external CO_2 in the range 3.8-13.6 mmol $\cdot$ m^{-3}. The time taken for external ^{14}C inorganic C equilibration with the intracellular pool of inorganic C is 30-90 min, and the time for assimilation of the intracellular inorganic C pool is 10-50 min. Berman-Frank and Erez (1996) suggested that the equilibration time for the intracellular inorganic C pool in microalgae (although only one of which was a dinoflagellate) increases with increasing cell size and decreasing surface area per unit volume. Such a long equilibration time on the intracellular inorganic C pool with increasing cell size is probably also consistent with long equilibrium times experienced in Symbodiniaceae cells within their coral hosts, e.g. coral *Styllophora pistillata* (Furla et al. 2000).

Isensee et al (2014; their Table 4 compilation) shows that the intracellular inorganic C concentration in *Peridininium gatunense* is the highest of the published values for cyanobacteria and eukaryotic microalgae. The three highest intracellular inorganic carbon concentrations (Isensee et al. 2014 are for *Peridinium gattunense* and two β-cyanobacteria with, respectively, Form II and cyanobacterial Form IB RuBisCO. Hopkinson et al. (2014) found an internal pool of inorganic C of 15 mol m^{-3} in the α-cyanobacterium *Prochlorococcus*

spp. MED4 in seawater with 2 mol m^{-3} inorganic C, using MIMS (membrane inlet mass spectrometry) to measure the inorganic C pool as the integrated post-illumination CO_2 efflux in excess of dark respiration. α-cyanobacteria have Form IA RuBisCOs which, like the Form IB RuBisCO of β-cyanobacteria and the Form II Rubisco of basal dinoflagellates, have greater $K_{0.5}$ and lower CO_2:O_2 selectivity than the Form IB RuBisCOs of the green line of algae and embryophytes, and the Form ID Rubiscos of the red line of algae (other than basal dinoflagellates; Raven et al. 2017, Bathellier et al. 2018; see also Nimer et al. 1999). Such patterns potentially explain the greater accumulation of inorganic C in the dinoflagellate, the α-cyanobacterium and the two β-cyanobacteria than in other algae with CCMs (Hopkinson et al. 2014; Table 4 of Isensee et al. 2014).

An aspect of the large intracellular inorganic C in concentration in *Peridinium gatunense* is the relation to limitation on total intracellular osmolarity of freshwater flagellate algal cells. The mean value of intracellular inorganic C, 112 mol · m^{-3}, in illuminated *Peridinium gattutense* correspondsto an osmolarity of 101 osmol · m^{-3}, assuming an osmotic coefficient of 0.9. It is likely that much of the intracellular inorganic C is HCO_3^- with charge-balancing cations, so the osmolarity of the intracellular inorganic C pool is > 101 osmol · m^{-3}. While no intracellular osmolarity values are available for freshwater dinoflagellates, a freshwater photosynthetic chrysophycean freshwater flagellate lacking a CCM has a total intracellular osmolarity of 75 osmol m^{-3} (Raven 1982, 2018). Low intracellular osmolarity in actually or effectively freshwater flagellates decreases the energy cost of volume regulation (Raven 1982, 2018), so increasing the intracellular osmolarity as in *Peridinium gatunense* could more than double the energy cost of volume regulation, at least in the light when the CCM is operating.

Nimer et al. (1999) further investigated inorganic C accumulation in the marine dinoflagellate *Prorocentrum micans* using the silicone oil centrifugation technique at an external pH of 8.3 and 2 mol · m^{-3} inorganic C. Intracellular inorganic C in the light was 10.5 mol · m^{-3}, over 5 times the external value. However, other data suggest smaller inorganic carbon, and CO_2 accumulation in marine microalgae than was found by Nimer et al. (1999): the variation of accumulation among laboratories and among species needs further clarification.

Leggat et al. (1999) measured inorganic C accumulation using ^{14}C-inorganic C and silicone oil centrifugation in two marine dinoflagellates, the free-living *Amphidinium carterae* and symbiotic Symbiodiniaceae cells from the giant clam *Tridacna gigas*. For *Amphidinium carterae* cultured at pH 7.0, the internal:external inorganic C ratio is 7.3-26.4 for external inorganic C concentrations of 20 – 200 mmol · m^{-3}. How these accumulation ratios relate to cells in nature with external inorganic C of about 2000 mmol · m^{-3} at pH of about 8 is currently unclear. For Symbiodiniaceae, freshly isolated cells were cultivated for 1 to 5 d and measured at pH 7.0 and at pH 8.0. For freshly isolated cells at pH 8.0 the external inorganic C concentrations used were 200, 400, 1000 and 2000 mmol · m^{-3}, and the internal:external accumulation ratio was 0.9 – 1.9. The accumulation ratio is 2.2 – 4.6 times that predicted from passive CO_2 diffusive equilibration. For all other experiments on Symbiodiniaceae cultured at pH 7 with 20-200 mol · m^{-3} the accumulation ratio was 1.5-7.0 times that expected for CO_2 passive diffusive equilibration. Again, it is not clear how these accumulation ratios relate to non-symbiotic cells in nature with external inorganic C of about 2000 mmol · m^{-3} at pH of about 8.

Ratti et al. (2007) investigated the CO_2-concentrating mechanism in the marine dinoflagellate *Protoceratium reticulatum* grown at 370 µmol CO_2 mol^{-1} total gas and 5000 µmol CO_2 · mol^{-1} total gas. The accumulation of inorganic C in cells grown at the lower CO_2 concentration is 1.5 fold that expected for passive diffusion of CO_2 according to the extracellular-intracellular pH difference, while there is no accumulation in cells grown at the higher CO_2 level.

Evidence from in vivo inorganic carbon affinity

The available evidence shows that most microalgae examined are inorganic carbon saturated for photosynthesis and growth in their natural environments (Clark and Flynn 2000, Colman et al. 2002, Giordano et al. 2005, Tatters et al. 2013, Raven and Giordano 2017, Raven et al. 2017). For example, the freshwater dinoflagellate *Peridinium gatunense* has the following $K_{0.5}$ values at the indicated pH values, pH 7.3, $K_{0.5}$ inorganic C = 800 mmol · m^{-3}, $K_{0.5}$ CO_2 = 112 mmol · m^{-3}; pH 8.3, $K_{0.5}$ inorganic C = 300 mmol · m^{-3}, $K_{0.5}$ CO_2 = 4 mmol · m^{-3}; pH 9.2, $K_{0.5}$ inorganic C = 90 mmol · m^{-3}, $K_{0.5}$ CO_2 = 0.1 mml · m^{-3} (Berman-Frank et al. 1998). Lake Kinnerett, from which the alga was obtained has pH in the range 8.0-9.2, and inorganic

C varies inversely with pH in the range 1.6 – 2.7 mol · m^{-3} (Berman-Frank et al, 1998). Consequently, inorganic C is probably always saturating for photosynthesis for *Peridinium gatunense* in Lake Kinnerett.

Nimer et al. (1999) found $K_{0.5}$ inorganic C = 750 mmol · m^{-3} at an external pH of 8.3 in the marine dinoflagellate *Prorocentrum micans* (i.e., inorganic C saturated for photosynthesis in seawater at 2 mol inorganic C · m^{-3}). Leggat et al. (1999) and Badger et al. (2000) grew a Symbiodiniaceae species isolated from *Tridacna gigas* to study gross and net photosynthetic rate and light-dependent O_2 uptake using ^{18}O-enriched O_2, as a function of inorganic C concentration. In seawater medium at pH 8 the $K_{0.5}$ for gross photosynthesis is 0.1mol inorganic C · m^{-3}, so photosynthesis is saturated with inorganic C in seawater.

A wide range of examples all similarly show that inorganic C is in fact routinely saturating in the natural environment. Clark and Flynn (2000) used pH drifts to determine the half-saturated concentration of inorganic C for growth of the marine dinoflagellates *Alexandrium fundyense* (0.47 mol · m^{-3}) and *Scrippsiella trochoidea* (0.28 mol · m^{-3}). Dason and Colman (2004) found $K_{0.5}$ in terms of inorganic carbon in seawater at pH 8 of 0.43 mol · m^{-3} for *Heterocapsa oceanica* and 0.56 mol · m^{-3} for *Amphidinium carterae*. Rost et al. (2006) examined the affinities (expressed as $K_{0.5}$ for HCO_3^-) in red tide marine dinoflagellates *Prorocentrum minimum* and *Heterocapsa triquetra* at external pH 8 and pH 9.1, to yield values of 0.15 (*P. minimum*) and 0.45 (*H. triquetra*) mol · m^{-3} at pH 8, and 0.01 (*P. minimum*) and 0.02 (*H. triquetra*) mol · m^{-3} at pH 9.1, i.e., photosynthesis is saturated with HCO_3^- at the concentration in seawater at the respective pH value. Finally, Søderberg and Hansen (2007) examined the effects of high pH and low inorganic C concentrations in three temperate marine species of *Ceratium.* pH drift (d) generally gave slightly lower maximum pH values for growth than did constant (c) pH experiments. The highest pH values for growth were 8.7 for both methods for *Ceratium fusus*, 8.8 (d) and 9.1 (c) for *Ceratium furca* and 8.5 (d) and 8.9 (c) for *Ceratium tripos.* For *Ceratium furca*, the CO_2 plus HCO_3^- concentration needed for half the maximum growth rate ($K_{0.5}$) at pH 8 was about 300 mmol · m^{-3}, i.e. saturated by 2 mol · m^{-3} inorganic C in seawater; at least 750 mmol (CO_2 + HCO_3^-) m^{-3} was needed for half maximum growth at pH 9. Finally, Eberlein et al. (2014) showed that, while both *Alexandrium tamarense* and *Scripsiella trochoidea* show photosynthetic rates independent of

the inorganic C concentration tested, there were significant differences in photosynthetic physiology as a function of inorganic C concentration between the two species.

All of the cases considered above have Form II RuBisCO. *Karenia brevis* has Form ID RuBisCO, and has significantly greater specific growth rate at both 25°C and 30°C with 1000 μmol · mol^{-1} CO_2 than with 350 μmol · mol^{-1} or 250 μmol · mol^{-1} (Errera et al. 2014). This contrasts with the findings of Bercel and Kranz (2019) on *Karenia brevis*, i.e., no significant difference in specific growth rate or photosynthetic rate with CO_2 at 150 μmol · mol^{-1}, 400 μmol · mol^{-1}or 780 μmol · mol^{-1}. *Karenia mikimotoi*, which also has Form ID RuBisCO, shows increasing specific growth rate, gross photosynthetic rate, dark respiration rate and RuBisCO activity per cell, with CO_2 concentration, using the mol fractions 400 μmol · mol^{-1}, 1000 μmol · mol^{-1}, 2000 μmol · mol^{-1}. The specific growth rate of *Karlodinum veneficium*, also with Form ID RuBisCO, is significantly higher at pH 8.03 than at pH 7.51 (Müller et al. 2019; i.e., the reverse of the effect seen in *Karenia brevis* when expressed in terms of CO_2 concentration). However, the two treatments are not identical; increased CO_2 decreases pH but increases total inorganic C, while decreasing pH increases CO_2 but does not alter total inorganic C.

In conclusion, dinoflagellates with Form I RuBisCO have more variable degrees of inorganic C saturation in their air-equilibrated natural environment than do dinoflagellates with Form II RuBisCO.

Form of inorganic C entering photosynthesising dinoflagellates

Methods for quantifying CO_2 and HCO_3^- influx in photosynthesis depend on the absence (or inhibition) of extracellular carbonic anhydrase, regardless of whether the MIMS or ^{14}C-CO_2 and $H^{14}CO_3^-$ isotope disequilibrium method is used (Briggs 1958, Dason et al. 2004, Rost et al. 2006, Brading et al. 2013, Hoins et al. 2016a). MIMS measures net changes in external O_2 concentration and external CO_2 concentration, and net HCO_3^- influx is determined as the difference between net O_2 evolution and net CO_2 uptake, assuming net (CO_2 + HCO_3^-) influx = net O_2 efflux (Badger et al. 1994, Rost et al. 2007); this assumption of net (CO_2 + HCO_3^-) influx = net O_2 efflux can be avoided by measuring net inorganic C influx (Badger et al. 1994, Rost et al. 2007). The isotope disequilibrium method suspending illuminated cells in inorganic C-free growth medium strongly buffered at the growth pH and ^{14}C-inorganic C as $^{14}CO_2$ (concentrated low pH solution) or $H^{14}CO_3^-$ (concentrated high pH solution) at time zero, and

sampling cells at intervals for ^{14}C-organic carbon. If the initial ^{14}C assimilation rate with $^{14}CO_2$ is higher than the steady state rate, and the initial ^{14}C assimilation rate with $H^{14}CO_3^-$ is lower than the steady state rate, then CO_2 is the major form of CO_2 entering. If the initial ^{14}C assimilation rate with $^{14}CO_2$ is lower than the steady state rate, and the initial ^{14}C assimilation rate with $H^{14}CO_3^-$ is higher than the steady state rate, then HCO_3^- is the major form of CO_2 entering. When there is extracellular carbonic anhydrase, the need to inhibit this enzyme by acetazolamide (LaPointe et al. 2008) or dextran-bound sulphonamide (Nimer et. 1999; but not ethoxyzolamide, which inhibits both extracellular and intracellular carbonic anhydrase; Dason et al. 2004), means that both techniques under-estimate CO_2 influx relative to the situation in which the extracellular carbonic anhydrase is active. Rost et al. (1994) reported that the MIMS and isotope disequilibrium methods gave the same results within experimental error.

Based on MIMS, CO_2 is the dominant inorganic C species entering *Amphidinium carterae* and *Heterocapsa oceanica* (Dason et al. 2004); using isotope disequilibrium, CO_2 is also the dominant form of CO_2 entering two strains of Symbiodiniaceae (Brading et al. 2013). By contrast, using both MIMS and isotope disequilibrium techniques, Rost et al. (2006) found that at least 80% of inorganic C entering *Prorocentrum minimum*, *Heterocapsa triquetra* and *Ceratium rubrum* was as HCO_3^- at seawater pH of both 8.0 and 9.1. Rost et al. (2007) used 50 mol m^{-3} HEPES (2-[4-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid) pH buffer in the MIMS experiments, and 20 mol m^{-3} BICINE (N,N-Bis(2-hydroxethyl) glycerine) pH buffer in the isotope disequilibrium experiments. The use of buffers means that the mechanism of HCO_3^- use based on localized surface acidification (Walker et al. 1980, Price and Badger 1985), is not a possibility for HCO_3^- use in the three dinoflagellates, since the buffer abolishes acid-base surface zonation. Furthermore, the acid-base zonation mechanism typically involves extracellular carbonic anhydrase, and this has a very low activity in the three dinoflagellates. It should also be pointed out that dinoflagellate cells are probably too small to support the necessary separation of acid and alkaline zones (Raven and Beardall 2016) that might otherwise be invoked. Hoins et al. (2016a) found that HCO_3^- entry comprised 19-53% of the total inorganic C uptake four species of dinoflagellate (*Alexandrium fundyensis*, *Scripsiella trochoidea*, *Gonyaulax spinifera* and *Protoceratium reticulatum*) in seawater equilibrated with atmospheres containing 180, 380, 800 and 1200 μmol · mol^{-1} total gas.

Hoins et al. (2016a) also measured leakage of CO_2 from the four dinoflagellates, finding 44-71% of the inorganic C that had entered leaked out. While Ratti et al. (2007) found (based on pH dependence) no significant evidence of HCO_3^- use in *Protoceratium reticulatum*, HCO_3^- has been observed to contribute 44-49% of the inorganic C influx for this dinoflagellate (Hoins et al. 2016a). Finally, Nimer et al. (1999) showed that inorganic C enters the marine dinoflagellate *Prorocentrum micans* as CO_2, based on the inhibition of inorganic C accumulation and of photosynthesis by the addition of the membrane-impermeant carbonic anhydrase DBS. Here, in contrast to the MIMS and isotope disequilibrium techniques for determining the inorganic C species entering where any external inorganic carbon must be inhibited, the presence of the extracellular carbonic anhydrase in control cells, and its inhibition in the treatment, is the basis for demonstrating entry of CO_2.

Again, all of the cases considered above are for cells with Form II RuBisCO. *Karenia brevis*, with Form ID RuBisCO, shows an increase in half saturation of photosynthesis expressed as CO_2, and, using isotope disequilibrium, increased CO_2 entry relative to HCO_3^-, with in increasing CO_2 (150, 400 and 780 $\mu mol \cdot m^{-3}$; Bercel and Kranz 2019). *Gymnodinium mikimotoi* (= *Karenia mikimotoi*) also has a Form I RuBisCO (Takishita et al. 2006), has only a small fraction of the total carbonic anhydrase activity outside the cell and insignificant inhibition of the relative electron transport rate (rETR), measured by chlorophyll fluorescence, by acetazolamide at all growth CO_3 concentrations (Hu et al. 2017), Also in *G. mikimotoi*, inhibition by the anion (HCO_3^-) transporter DIDS is small and decreases at the higher growth CO_2 concentrations (Hu et al. 2017), consistent with little HCO_3^- entry that is decreased at high CO_2 for growth.

In conclusion, the mechanisms of CO_2 and HCO_3^- influx at the dinoflagellate plasmalemma are not well known. There are no well-authenticated examples of active transport of CO_2 across the membranes of any photosynthetic organism, so CO_2 accumulation in CCMs when CO_2 is the form crossing the membrane involves some energized process within the cell (Raven and Beardall 2016). The diffusive CO_2 influx through the lipid bilayer under a given driving force of CO_2 concentration difference can be increased by the presence of CO_2-permeable channels, e.g., CO_2-selective aquaporins (Raven and Beardall 2016). While there is evidence of aquaporins in dinoflagellates (e.g., Ryan et al. 2014), there is no evidence

on CO_2 selectivity or plasmalemma location. HCO_3^- influx cannot occur by passive entry through the lipid bilayer due to (1) the low permeability of ions and (2) the inside-negative electrical potential across the plasmalemma (Raven and Beardall 2016). Active transport of HCO_3^- at the plasmalemma of diatoms can involve DIDS-inhibited SLC4 transporters (Nakajima et al. 2013); SLC4 genes occur in dinoflagellates (Lin et al. 2015).

Inhibition of growth and photosynthesis at high (and low) pH

Studies of extremes of pH on growth and photosynthesis can give information on the capacity to photosynthesise and grow at extremely low concentrations of CO_2 (high pH) or of HCO_3^- (low pH), with low pH (<6.5) limited to inland waters such as poorly inorganic C-buffered bog pools, flooded open-cast lignite mines and, particularly, acidic hot springs (pH < 3). However, it is also known that extremes of environmental pH per se can limit growth and photosynthesis to a smaller pH range that would otherwise be determined by pH-related changes in the concentration of the different species of inorganic C available to the cells, with ecological consequences (Raven et al. 2020). Spilling (2007) found that a dense sub-ice bloom of brackish water (Baltic) dinoflagellates at low light and low temperatures was potentially limited by high pH (9.0). Previously, Hinga (1992; see also Hinga 2002) showed that marine dinoflagellates occurred at higher pH values than did diatoms in marine enclosures. This is consistent with a capacity of dinoflagellates to photosynthesise at lower CO_2:HCO_3^- than diatoms, but a direct pH effect has not been ruled out.

Nimer et al. (1997) subjected the marine *Glenodinium foliaceum* to pH drift, yielding a final pH of the very high value of pH 10.7 from the starting value of pH 8.3, and a decrease in inorganic C from 2.0 to 1.21 mol · m^{-3}, strongly indicating HCO_3^- active influx. The pH drift results were unaltered by the presence of DIDS (4'4'-diisothiocyanostilbene-2,2-disulfonic acid, an inhibitor of an anion exchanger), or by an inhibitor (DBS = dextran-bound sulphonamide) of extracellular carbonic anhydrase (as expected from the absence of detectable activity of this enzyme in *Glenodinium foliaceum*): Nimer et al. (1997). The very high pH achieved means that, even if the final pH was determined by a direct influence of pH effect, the conclusion on active HCO_3^- influx is sound.

Hansen (2002) further investigated the pH dependence of growth of three species of marine dinoflagellates, and found that the maximum pH for growth was 8.8 for *Ceratium*

lineatum, 9.4 for *Heterocapsa triquestra*, and 9.6 for *Prorocentrum minimum*, and discusses effects of these pH tolerances on species succession (see also Pedersen and Hansen 2003a, Berge et al. 2012). Havskum and Hansen (2006) found that cultures of *Heterocapsa triquetra* could increase the external pH to 9.55. The non-photosynthetic (phago-organotrophic) marine dinoflagellate *Oxyrrhis marina* was maintained up to the pH 9.9, the highest pH tested (Pedersen and Hansen 2003b), showing that when inorganic C acquisition is not a factor at least one species of dinoflagellate can maintain high growth rates at high pH.

Hansen et al. (2007) examined three species of marine dinoflagellates, showing a high affinity for inorganic C expressed as CO_2 during growth at pH 8, but a lower affinity at higher pH values of pH 8.55 (*Ceratium lineatum*) or pH 9.1 (*Heterocapsa triquetra* and *Prorocentrum minimum*). The upper pH limit is an effect of limitation by high pH rather than by low inorganic C (Hansen et al. 2007); limitation by CO_3^{2-} inhibition rather than OH^- inhibition cannot be ruled out on the data provided, or those in Søderberg and Hansen (2007) on three marine species of *Ceratium*. Hu et al. (2017) found the maximum pH of 9.8 in pH drift using *Karenia mikimimotoi*, indicating HCO_3^- use.

Less effort has been expended in studying the lower pH limit of growth of dinoflagellates. Dason and Colman (2004) showed that the growth rate of the marine dinoflagellates *Amphidinium carterae* and *Heterocapsa oceanica* grew less rapidly in air-equilibrated seawater at pH 7 than at pH 7.5, 8.0 or 8.5. Dason and Colman (2004) also determined the intracellular pH of both dinoflagellates at external pH values of 7.0, 7.5 and 8.5, and found close to a 1:1 ratio of internal to external pH, in contrast to most other microalgae examined where the internal pH changes by 0.1 to 0.5 units for a 1 unit change in external pH (Raven 2013).

Hill et al. (2019) examined Symbiodiniaceae sp. isolated from the coral *Mussismilia braziliensis*, and cultured *ex hospite* for 16 days under control (pH 8.16, 484 μmol $CO_2 \cdot$ mole atmosphere^{-1}) and acidified (pH 7.59, 1633 μmol $CO_2 \cdot$ mol atmosphere^{-1}) conditions. Cells grew in the control cultures, but there was no growth in the acidified cultures; photosynthetic capacity as estimated from PSII fluorescence was decreased by 40% in the acidified treatment relative to the controls (Hill et al. 2019). The extent to which the inhibition is a result of the changed pH, of the change in inorganic C, or of both factors, was not examined.

In conclusion, more work is needed to determine the upper and, especially, lower pH limits of photosynthesis and growth, and whether the limits are related to pH per se, to changes in inorganic C, or both.

Occurrence of pyrenoids

Pyrenoids are structures in the stroma of many eukaryotic algae that generally contain most of the cell quota of RuBisCO, and their presence in microalgae is generally correlated with the occurrence of CCMs, although CCMs can occur in eukaryotic algae lacking pyrenoids (Meyer et al. 2017, Raven and Giordano 2017). Dodge (1968) and Dodge and Crawford (1971) examined the fine structure of pyrenoids in dinoflagellates; pyrenoids occur in half of the thirty or so species examined. Seo and Fritz (2002) examined the occurrence of pyrenoids through the diel light-dark cycle in two freshwater and two marine dinoflagellates; in all cases pyrenoids were found in the photoperiod but not in the scotoperiod. Jenks and Gibbs (2001) used immunological techniques to examine the distribution of Form II RuBisCO in chloroplasts of *Amphidinium carterae*: RuBisCO occurs mainly in the pyrenoid, with some in the stroma. Similarly, Nassoury et al. (2001) found that Form II RuBisCO was predominantly in pyrenoids of *Gonyaulax polyedra* when CO_2 assimilation rates were high, and dispersed in the stroma at other times. Ratti et al. (2007) could not find a pyrenoid under any of the growth conditions used for *Protoceratium reticulatum*, although Hansen et al. (1996/1997) found a simple pyrenoid in this species. Form II containing Symbiodiniaceae have been commonly observed to house RuBisCO within stalked pyrenoids (e.g., Blank 1987). Meyer et al (2017), in discussing what is known, and what remains to be discovered, about the algal pyrenoid, point out that the pyrenoids of dinoflagellates are usually penetrated by thylakoids or thylakoid tubules. Whether these contain carbonic anhydrase in their lumen, and so function as in the chlorophycean *Chlamydomonas reinhardtii* and the diatom *Phaeodactylum tricornutum* is not known (Meyer et al. 2017, Mukherjee et al. 2017).

In conclusion, with the background that pyrenoids are not essential for eukaryotic CCMs, and are occasionally found (presumably as evolutionary relics) in algae that have no detectable CCM, the occurrence of approximately equal number of photosynthetic dinoflagellate species with and without thylakoids is not surprising. Where pyrenoids occur,

more work is needed on the extent to which RuBisCO and intracellular carbonic anhydrases occur in the pyrenoids, and the occurrence and function of thylakoids in pyrenoids. It would also be useful to determine how pyrenoid occurrence relates to the form of RuBisCO.

Carbonic anhydrase

External carbonic anhydrase

For cells that express Form II RuBisCO, and when CO_2 is the species of inorganic C entering the cell and HCO_3^- is the main inorganic C species in an air-equilibrium medium, external carbonic anhydrase (CA) activity can increase CO_2 supply to the outer surface of the organism by restoring the CO_2:HCO_3^- ratio at the inner surface of the DBL as CO_2 is removed by the photosynthesising cells. External CA activity in the freshwater *Peridinium gatunense* increases when inorganic C is limiting the rate of photosynthesis (Berman-Frank et al. 1994, Berman-Frank et al. 1995) although the form of inorganic C entering the cells is not clear. Nimer et al. (1997) measured the extracellular CA activity of five species of marine dinoflagellates grown under inorganic C-replete and inorganic C-limited. Four species (*Amphidinium carterae*, *Prorocentrum micans*, *Prorocentrum minimum* and *Scrippsiella trochoidea*) had significant external CA activity in inorganic C-replete cultures with higher activity in inorganic C-limited cultures. *Glenodinium foliaceum* had no detectable external CA activity: this species was (unlike the other dinoflagellates) subjected to pH drift yielding a final pH of the very high value of 10.7 from the starting value of 8.3, and a decrease in inorganic C from 2.0 to 1.21 mol $\cdot$ m^{-3}, strongly indicating HCO_3^- active influx. The pH drift results were unaltered by the presence of the anion transport inhibitor DIDS, or by DBS, an inhibitor of extracellular CA (as expected from the absence of detectable activity of this enzyme in *Glenodinium foliaceum*).

Nimer et al. (1999) also showed that the marine dinoflagellate *Prorocentrum micans* has an extracellular CA, inhibition of which by DBS completely inhibited inorganic C accumulation and photosynthesis, demonstrating that CO_2 is the inorganic C species entering the cells. Dason et al. (2004) measured low activities of external CA in the marine dinoflagellates *Amphidinium carterae* and *Heterocapsa oceanica* by the potentiometric technique, but negligible activity using MIMS. Ratti et al. (2007) found no external CA in *Protoceratium reticulatum*, a marine alga for which pH drift indicates little use of external

HCO_3^-. In contrast, Lapointe et al. (2008) found an external δ-CA in the marine *Lingulodinium polyedrum*, an alga for which CO_2 appears to be the dominant inorganic C species taken up.

For *Karenia brevis* with Form ID RuBisCO, external CA activity is significantly higher in cells grown at 150 μmol CO_2 mol^{-1} total gas than when grown at 400 or 780 μmol CO_2 · mol total gas^{-1} (Bercel and Kranz 2019). *Karenia mikimotoi*, also with Form ID Rubisco, shows no significant difference in external CA activity with growth CO_2 in the range 400 μmol CO_2 · mol total gas^{-1}, 1000 μmol CO_2 · mol total gas^{-1}, 2000 μmol CO_2 · mol total gas^{-1}.

In conclusion, there is incomplete correlation between the occurrence of external carbonic anhydrase and CO_2 as the inorganic C source entering the cells for dinoflagellates with Form II Rubisco. For dinoflagellates with Form ID Rubiscos the expression of extracellular carbonic anhydrase shows variable responses to low inorganic C supply.

Internal carbonic anhydrase

Intracellular CA activity is needed for all known CCMs. Although CO_2 is the inorganic C species used by RuBisCO, there is no convincing evidence of active transport of CO_2 that could explain intracellular accumulation of CO_2 (Raven and Beardall 2016). One mechanism of cyanobacterial intracellular accumulation of HCO_3^- is based on energetically downhill entry of CO_2 into the cytosol, followed by conversion of CO_2 to HCO_3^- energized by an Ndh (NAD(P)H: quinone oxidoreductase) thylakoid component. This mechanism has no known analogue in eukaryotes: the CA-like protein associated with Complex I (Ndh-like) of algal and plant mitochondria has no known CA activity, and mitochondrial Complex I is absent from dinoflagellates and chromerids (Raven and Beardall 2017).

The effects of CO_2 availability on CA activity during the annual bloom of the dinoflagellate *Peridinium gatunense* in the freshwater Lake Kinnerett (= Sea of Galillee, where HCO_3^- is 97% of total inorganic C) is that CO_2 limitation is correlated with increases in external and internal CA activity (Berman-Frank et al. 1994). Ratti et al. (2007) investigated the marine dinoflagellate *Protoceratium reticulatum* and found that the intracellular β-CA activity, calculated to be an essential component of the CCM, is decreased 60% in cells grown at a higher CO_2 concentration. However, the most comprehensive data set on intracellular CA in free-living dinoflagellates to date is for the three bloom-forming red tide species *Ceratium lineatum*, *Heterocapsa triquetra* and *Prorocentrum minimum* acclimated to pH 8.0, and to

either pH 8.5 (*Ceratium lineatum*) or pH 9.1 (*Heterocapsa triquetra* and *Prorocentrum minimum*), the highest pH values at which the cell division rate was not decreased relative to the value at pH 8.0 (Rost et al. 2006). MIMS showed very low activities of intracellular carbonic anhydrases, and only in the case of *Prorocentrum minimum* did intracellular CA activity increase at the higher growth pH (Rost et al. 2006). *Karenia mikimotoi*, with Form ID RuBisCO, shows decreased internal CA activity with growth CO_2 through the range 400 μmol CO_2 · mol total gas^{-1}, 1000 μmol CO_2 · mol total gas^{-1}, 2000 μmol CO_2 · mol total gas^{-1}.

In conclusion, all eukaryotic CCMs require intracellular carbonic anhydrases. Dinoflagellates with Form II and Form ID RuBisCOs show increased intracellular carbonic anhydrase expression with growth at low inorganic C concentrations. Howveer, more information is needed on the location intracellular carbonic anhydrase and of intracellular inorganic C transport.

Natural abundance stable carbon isotope discrimination

The difference between the natural abundance of the stable ^{13}C :^{12}C of organic C of a photosynthetic organism and that of the inorganic C available to the organism during its growth can yield information about the inorganic C source (CO_2 or HCO_3^-) entering the cell as well as the occurrence of a CO_2 concentrating mechanism (Zeebe and Wolf-Simon 2001, Raven et al. 2002). The global ^{13}C:^{12}C is 1.1:98.9 (Zeebe and Wolf-Gladrow 2001); the ^{13}C:^{12}C of the sample is usually expressed using the δ notation relative to a standard of accurately known ^{13}C:^{12}C, i.e., the Vienna Pee-Dee Belemnite (VPDB), thus $\delta^{13}C = [\{(^{13}C:^{12}C)_{sample} / (^{13}C:^{12}C)_{VPDB}\}-1] \times 1000$, units of ‰ (parts per thousand). At 20°C, HCO_3^- dissolved in seawater has a $\delta^{13}C$ of 0‰, and CO_2 dissolved in seawater has a $\delta^{13}C$ of -10‰. The carboxylation enzyme Rubisco of the Calvin-Benson-Bassham cycle has some phylogenetic variation in the $\delta^{13}C$ of the organic products relative to its inorganic C substrate, CO_2, and account needs to be taken of the minor (~5%) of gross photosynthetic inorganic C fixation contributed by anaplerotic carboxylations (Raven et al. 2002). With no constraints on CO_2 supply, the $\delta^{13}C$ of the organic product is ~27‰ more negative than source CO_2 for C_3 photosynthetic biochemistry; this difference is denoted by Δ (≈ ε_p of some authors: Zeebe and Wolf-Gladrow 2001). CO_2 diffusion in water, and though membranes, has very small C isotope discrimination, so, with diffusive CO_2 entry, greater diffusive limitation of

photosynthesis means a smaller Δ value (Raven et al. 2002). The other major factor decreasing Δ is CCMs involving HCO_3^- influx and/or C_4 photosynthetic biochemistry; in the absence of CO_2 leakage from photosynthesising cells, CCMs can yield positive Δ values. (Raven et al. 2002), Increasing fractions of leakiness of CCMs yield more negative Δ values (Raven et al. 2002, Raven and Beardall 2016).

Burkhardt et al. (1999a) and Burkhardt et al. (1999b) show that the stable C isotope fractionation ε_p increased as a function of CO_2 concentrations from 1 to 39 μmol · kg^{-1} for growth in marine phytoplankton, including the dinoflagellate *Scrippsiella trochoidea.* Hoins et al. (2016b) examined carbon stable isotope fractionation in four species of marine dinoflagellates, i.e., *Alexandrium fundyense*, *Scrippsiella trochoidea*, *Gonyaulax spinifera* and *Protoceratium reticulatum*, grown at 180-1200 μmol CO_2 · mol total gas^{-1}. For *Alexandrium fundyense* and *Scrippsiella trochoidea* discrimination between ^{13}C and ^{12}C increases with CO_2 concentration for growth, as predicted for an increased fraction of CO_2 as the inorganic C species entering the cell, and close to the predictions of a model of Hoins et al. (2016a). For *Gonyaulax spinifera* and *Protoceratium reticulatum*, the effects of CO_2 increase on C isotope fractionation are more variable, so the fit to the model is less good (Hoins et al. 2016b). The isotope fractionation also depends on the fractional CO_2 leakage from the cells (Raven and Beardall 2016); leakage increases with the CO_2 concentration and with increasing isotope fractionation in *Alexandrium fundyense*; for the other three species tested the fractional CO_2 leakage is more or less independent of the CO_2 concentration for growth (Hoins et al. 2016a). Hoins et al. (2016b) extended this work to include the interaction of light and nitrogen availability on the C isotope fractionation in the four dinoflagellate species, and found that for the two species tested there was no change in the CO_2 dependency of C isotope fractionation with growth at high or low light, while for the other two species the CO_2 dependency of C isotope fractionation disappeared when nitrogen supply was growth limiting. As is often the case, studies of natural abundance of stable C isotopes in organisms is best considered in the context of other data; for dinoflagellates with Form II Rubisco there is the additional problem of not knowing the in vitro inorganic C ^{13}C:^{12}C discrimination of the Form II Rubisco of dinoflagellates owing to their very short active catalytic time in vitro.

Wilkes et al. (2017) investigated the CO_2 dependence of C isotope fractionation in the marine dinoflagellate *Alexandrium tamarense*, and found, as shown by Hoins et al. (2016a,b)

for *Alexandrium fundyense* and *Scrippsiella trochoidea*, increasing C isotope fractionation with increasing CO_2 concentration for growth. Wilkes et al. (2017) use the three data sets to predict the intrinsic stable C isotope fractionation of the dinoflagellate variant of Form II Rubisco relative to the source CO_2 (ε_p) of 27‰, which is not significantly different from the 25‰ ε_p estimated by the same method for (non-dinoflagellate) marine phytoplankton with Form ID Rubisco (Wilkes et al. 2017). As noted above, the fragility of the dinoflagellate Form II RuBisCO in vitro (e.g., Whitney and Andrews 1998) means that the present means of measuring C isotope fractionation in vitro (e.g., McNevin et al. 2007) cannot be used on these dinoflagellate Form II enzymes. In vitro measurements of the C isotope fractionation Form II RuBisCOs from bacteria give ε_p values significantly lower (ε_p = 22.2‰ for the *Rhodospirillum rubrum* enzyme; McNiven et al. 2007) than the 27‰ estimated in vivo (Wilkes et al. 2017). Form II RuBisCOs are not unique in this mismatch between in vitro and in vivo estimates of $\varepsilon_{p;}$; this is also the case for Form ID RuBisCO in diatoms and haptophytes (Boller et al. 2011, 2015, Wilkes et al. 2017).

What can the natural abundance of stable carbon isotopes tell us about inorganic C acquisition in dinoflagellates? Interpretation of the natural abundance of stable C isotopes in dinoflagellates with Form II RuBisCO as a function of environmental conditions is restricted by the absence of measurements of C isotope discrimination by the enzyme in vitro as a consequence of the active lifetime of the enzyme. The observed increase in fractionation with increasing inorganic C supply for growth can be interpreted either by decreased transport limitation with diffusive CO_2 entry, or by replacement of a CCM by diffusive CO_2 entry. As is usually the case with natural abundance of stable isotopes, they are most useful in combination with other data.

Mechanisms of active transport of inorganic C

As indicated above, there is no convincing evidence of active transport of CO_2 in any organism studied to date. The anion exchange inhibitor DIDS does not inhibit the pH drift, whose extent probably relates to HCO_3^- entry, in the marine *Glenodinium foliaceum* (Nimer et al. 1997). SLC4 HCO_3^- transporters are known from the genomes of a species of the (present) Symbioniaceae (Aranda et al. 2016), *Alexandrium monilatum* and *Prorocentrum minimum* (Hennon et al. 2017) and *Thoracosphaera heimii* (Van de Waal et al. 2013); presumably these

are expressed in the plasmalemma. The various SLC4 HCO_3^- transporters can be Na^+:HCO_3^- symporters or HCO_3^-:Cl^- antiporters; work on the dinoflagellate SLC4 is needed to determine the co- or counter-ions, and their stoichiometry (Liu et al. 2015, Raven 2020, Raven and Beardall 2020), and hence the possibility that this transporter brings about active HCO_3^- influx with corresponding accumulation of HCO_3^- in the cytosol. For the organisms in which CO_2 is a major inorganic C species entering the cell, and possibly when HCO_3^- enters the cell, active HCO_3^- influx into the plastid is needed. This possibility seems not to have been explored in dinoflagellates in the way that it has been in diatoms (Hopkinson et al. 2011). Such studies would be facilitated by the isolation of pure, metabolically active, plastids. So far no such preparations have been reported for dinoflagellates (Laatsch et al. 2004, Wang et al. 2005) or chromerids (Sharaf et al. 2019).

Conclusions on inorganic carbon acquisition by free-living dinoflagellates

Basal dinoflagellates (and chromerids) have Form II RuBisCO acquired by horizontal gene transfer from a proteobacterium. Subsequent endosymbiotic gene transfer, endosymbiosis, and kleptoplasty replaces Form II RuBisCO in a minority of dinoflagellates with Form IB RuBisCO from cyanobacteria, Form IB RuBisCO from green algae, or Form ID RuBisCO from diatoms or haptophytes (Table 1). The short in vivo lifetime of the enzyme has led to limited analysis of dinoflagellate Form II RuBisCO kinetic properties to measuring the CO_2:O_2 selectivity, which resemble the values for proteobacterial Form II RuBisCOs. Assuming the other kinetics of the dinoflagellate Form II RuBisCO are similar to those of the proteobacterial enzyme, and the observed RuBisCO content of the cells, a CCM is needed for photosynthesis in air-equilibrium media. A CCM is also needed for dinoflagellates with cyanobacterial Form IB RuBisCO, and even dinoflagellates with green algal Form IB, and Form ID RuBisCO. The limited data on short-term ^{14}C-inorganic C assimilation show that dinoflagellates with Form II RuBisCO have the C_3 pathway of autotrophic organic C assimilation, so they do not have a C_4 biochemical CCM. Measurements of intracellular inorganic C concentration in photosynthesising dinoflagellates yielded a wide range of values, almost all showing concentrations in excess of those in the medium; *Peridinium*, the alga with the highest intracellular concentrations, has an as yet unexplained slow equilibration of internal and external concentrations.

The inorganic C affinity in vivo of dinoflagellates with Form II or Form IB RuBisCO grown at present-day air equilibrium CO_2 concentrations are such that photosynthesis and growth are very generally saturated at present CO_2 concentrations. There are variable effects on inorganic C affinity of growth at CO_2 higher than the present values. Studies of the form of inorganic C entering the cells show that both CO_2 and HCO_3^- enter cells, with an increased fraction entering as CO_2 when grown at CO_2 concentrations higher than the current atmospheric level. These measurements involve inhibition of any extracellular CA activity; since many dinoflagellates with Form II or Form ID RuBisCOs have extracellular CAs, this complicates interpretation of experiments on the form of inorganic C entering the cells. Studies of the natural abundance of stable C isotopes have given little information on inorganic C acquisition in dinoflagellates. The extent to which a dinoflagellate increase the medium pH in the light, or grow and photosynthesise at high pH, can be used to indicate entry of HCO_3^-, although direct inhibition at high pH could confuse interpretation. Less information is available on any lower pH limit for photosynthesis and growth. Pyrenoids occur in the plastids of many dinoflagellates, and in the case examined (Jenks and Gibbs 2001, Nassoru et al. 2001) the pyrenoid contains most of the cell complement of Form II RuBisCO. It is not clear in which compartment(s) the intracellular CA is expressed in the dinoflagellates with Form II and Form IB RuBisCOs. Finally, the mechanism of active transport of inorganic C is not well understood, although inhibition by DIDS, and expression of SLC4, presumably relate to HCO_3^- influx at the plasmalemma. Since no active transport mechanism for CO_2 has been clearly established in any organism, accumulation of inorganic CO_2 in the pyrenoid, or more generally in the stroma, must involve active transport of HCO_3^- at the chloroplast envelope (or rely on H^+ pumping into the thylakoid: Mukherjee et al. 2019) with CA activity in the cytosol and chloroplast.

Inorganic C acquisition by symbioses with dinoflagellates as photobionts

By far the most widespread symbiotic dinoflagellates are members of the warm-water marine family Symbiodiniaceae (Leggat et al. 2005, Venn et al. 2008, Mayfield et al. 2014, Lee et al. 2015, Lajeunesse et al. 2018), which are symbiotic in Foraminifera (Pochon and Pawlowski 2006, Fay et al. 2009, Vogel and Uthicke 2012), Ciliata (Lobban et al. 2002, Mordret et al. 2016), Porifera (Hill et al. 2011), Acoela (Mendes-Lopez and Silveira 1994,

Hikosaka-Katayama et al. 2012), Cnidaria (Gattuso et al. 1999, Coonan et al. 2013, Klein et al. 2017, Raven et al. 2019), gastropod Mollusca (Burghardt and Wägele 2004) and bivalve Mollusca (Leggat et al. 2000, 2005). Free-living cells of Symbiodiniaceae are known from coastal waters, and have recently been shown to grow in the open ocean (Decelle et al. 2018). In the case of the acoel worm *Warinoa* there is *Amphidinium* as a photobiont as well as a member of the Symbiodiniaceae (Hikosaka-Katayama et al. 2012), while the acoel worm *Amphiscolops* has *Amphidinium* as the sole photobiont (Mendes-Lopez and Silveira 1994). In Radiolaria (see Sierra et al. 2013) the symbiotic dinoflagellates are not closely related to the Symbiodiniaceae (Swanberg 1983, Dolven et al. 2007, Gottschling and McLean 2013, Probert et al. 2014, Yuasa et al. 2016). This diversity of dinoflagellate symbionts is also the case for Acantharia (Decelle et al. 2012, Decelle 2013, Sierra et al. 2013). It is not clear if chromerids (basal to non-photosynthetic apicomplexans) are mutualistic symbionts of the corals with which they associate (Chakravarti et al. 2019). Other alveolates associated with corals are non-photosynthetic apicomplexans that retain four chlorophyll synthesis genes (Kwong et al. 2019).

In all cases where dinoflagellates are the photobiont (inhabitant), their cells are within the host (exhabitant) organism (Law and Lewis 1983): in the diverticula of the gut in bivalve gastropods, and intracellular in all other cases. Since external inorganic C must move through host membranes and cytosol to reach the photobiont inhabitant, the exhabitant could play a role in concentrating inorganic C round the photobiont (Raven 1993).

The range of exhabitants for dinoflagellate symbioses are listed in Table 2, along with the number of membranes between the medium and the plasmalemmas of the dinoflagellates. The greater the number of membranes, the more complex is the task of deciphering the mechanism of inorganic C transport to the photobiont.

Cnidarians

The Symbiodiniaceae symbionts of cnidarians occur in the oral endoderm (e.g., fig. 3B of Gattuso et al. 1999; fig. 8 of Furla et al. 2000). Despite the proximity of the photobiont-containing cells to the coelenteron the schemes of inorganic C supply to the photobionts of Gattuso et al. (1999) and Furla et al. (2000) only show inorganic C flux to the photobionts from the bulk seawater via the oral ectoderm and mesogloea. This is justified by the data of

Agostini et al. (2009) and Agostini et al. (2012) showing that the coelenteron is almost completely isolated from the surrounding seawater via the mouth, based on the higher concentration of vitamin B_{12} and of nitrate, nitrite, ammonium and phosphate than in seawater, and the coelenteron microbiome relative to that of seawater. Accordingly, the focus is on inorganic C flux from the bulk seawater via the aboral ectoderm and the mesogloea to the photobionts in the oral endoderm.

Raven (1981) and Raven et al. (2019; see also James and Larkum 1996) tabulates the maximum net photosynthetic rates on an area basis for a phylogenetic range of submerged aquatic macrophytes. The order of rates are: symbiotic corals > marine brown, green and red alga and seagrasses > freshwater submerged flowering plants > freshwater macroalga *Chara.* Davies (1977) and Narubini and Davies (1996), both recalculated into SI units following Raven (1981), found light-saturated net photosynthetic rates of about 5 μmol O_2 or $CO_2 \cdot$ m surface area$^{-2} \cdot s^{-1}$ for species of coral (*Montastrea, Montipora* and *Porites*). The central question here is whether CO_2 diffusion from the bulk phase to the coral surface account for the observed rate of photosynthesis? The initial assumption is that there is no uncatalysed CO_2-HCO_3^- interconversion (see Briggs 1958; the absence of uncatalysed CO_2-HCO_3^- interconversion is not realistic, but it has very little quantitative effect in this case) or catalyzed CO_2 –HCO_3^- interconversion between the bulk phase and the coral surface. Calculating the maximum diffusion boundary layer thickness (DBL; assuming zero CO_2 at the coral surface) we use a diffusion coefficient for CO_2 at 25°C of $2.10 \cdot s^{-1}$, 12 mmol $\cdot$ m^{-3} CO_2 in the bulk phase at 25°C in equilibration with a gas phase of 400 μmol $CO_2 \cdot$ mol total gas^{-1} (table 5.1 of Raven 1984), the observed photosynthetic rate of 5 μmol O_2 or $CO_2 \cdot$ m surface area$^{-2} \cdot s^{-1}$ gives a maximum DBL thickness of 4 μm. The observed DBL thickness value is 2 mm (Sashar et al. 1996), so CO_2 diffusion cannot supply corals' inorganic C needs. While the diffusion boundary layer over the fragments used in the gas exchange measurements is presumably less than that found in massive corals in nature, a diffusion boundary layer thickness of 4 μm for fragments is implausible; a calculation that was similarly concluded by Tansik et al. (2015). The conclusion from these calculations is that inorganic C diffusion to the surface of symbiotic corals occurs in the form HCO_3^-.

Crawley et al. (2009) and Comeau et al. (2017) examined inorganic C dependence of photosynthesis and (photo-)respiration in corals, while Noonan et al. (2013) showed that

long-term exposure to elevated CO_2 had no effect on the diversity of Symbiodiniaceae in scleractinian corals. Prolonged exposure to elevated CO_2 stimulates photosynthesis of growth of Symbiodiniaceae in the sea anemone *Anthopleura elegantissima* (Towanda and Thueson 2012) in laboratory cultures. This is also the case for the sea anemone *Anemonia viridis* growing at different distances from a shallow water CO_2 vent near the island of Vulcano in the Mediterranean (Suggett et al. 2012). For this long term 'natural experiment' there is the possibility that the inhabitant genotypes change as a function of distance from the vent; Suggett et al (2012) showed that the same genotype dominated along the CO_2 and pH gradient from vent to the un-influenced benthic habitat. The response of the uncalcified sea anemones to ocean acidification differs from the calcified corals in not being constrained by inhibition of calcification. Shorter-term work on the sea anemone *Aiptasia pulchella* (Weis 1991) showed that photosynthesis is saturated at about twice the seawater inorganic C, and the photosynthesis is inhibited by more than 50% at all inorganic C concentrations added to C-free seawater by the allegedly membrane impermeable carbonic anhydrase inhibitor acetazolamide. However, cytoimmunochemistry shows the dominant carbonic anhydrase activity is within animal cells close to the vacuole enclosing the symbiont, i.e. allegedly unavailable to inhibition by acetazolamide.

While Goiran et al. (1996) suggest that spontaneous dehydration of HCO_3^- is sufficient to support photosynthesis in *Galaxea fascicularis*, their calculation referred to the whole volume of the external medium rather than what is within 'diffusion range' of the coral fragments; the calculation is more suited to suspended microalgae than to macrophytes. As indicated below, Al-Moghrabi et al. (1996) showed 50% inhibition of photosynthesis by inhibition of external CA, suggesting that uncatalysed HCO_3^- to CO_2 conversion in the DBL is not sufficient for CO_2 supply for photosynthesis (or that the CA inhibitor is not specific).

Al-Moghrabi et al. (1996; see also Allemand et al. 1998) showed inhibition of photosynthesis by 50% when extracellular CA is inhibited by membrane impermeable AZA (acetazolamide), and by 100% when both intra- and extracellular CA is inhibited by EZA (ethoxyzolamide), in the coral *Galaxea fascicularis.* The same authors also showed 100% inhibition of photosynthesis by the anion transport inhibitor DIDS (but only 50% by the anion transport inhibitor SITS: disodium 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulphonate); there was also 50% inhibition by replacing Na^+ with $choline^+$. Aside from the

100% inhibition by DIDS in the work of Al-Moghrabi et al. (1996) the inhibitor effects suggests that half of the inorganic C influx is as CO_2 (probably, but not necessarily, by diffusion!) and the rest by an HCO_3^- transporter. It is unfortunate that the minus Na^+ treatment was not combined with the AZA, EZA, DIDS and SITS treatments, so it is not certain that the minus Na^+ effect relates to a HCO_3^- transporter. Bertucci et al. (2013) review findings on the occurrence and roles of CAs in anthozoan corals, showing that dinoflagellate-associated cnidarian species had a mean 29-fold greater CA activity than did aposymbiotic cnidarians. As little as 20% of the CA activity in symbiotic cnidarians is associated with the animal rather than the dinoflagellates (Bertucci et al. 2013).

Finally, Tansik et al. (2015) used MIMS to show that there was an extracellular CA in three Caribbean coral species (*Orbiculata faveolata*, *Porites astreoides* and *Siderastrea radians*) with an activity supplying CO_2 faster than the rate of photosynthesis, and that inhibition of this extracellular CA inhibited photosynthesis by about 50%. Tansik et al. (2017), using the same three corals, investigated further the external CA of the coral and the internal CA of freshly isolated symbionts. MIMS measurements were made of O_2 release, and uptake of $^{13}CO_2$ from a medium with labelled with ^{13}C inorganic C and buffered at pH 8.3 with 20 mol · m^{-3} TRIS. The $K_{0.5}$ for dissolved inorganic carbon and P_{max} of intact colonies, based on net O_2 evolution, are lower for *Orbiculata faveolata* than for the other two corals; the reverse is the case for external CA activity. For freshly isolated Symbiodiniaceae the $K_{0.5}$ values are about an order of magnitude lower than for whole colonies. Figure 4 of Tansik et al. (2017) presents a model of transporters involved in supply of inorganic C for photosynthesis, and the inorganic C concentrations in the perisymbiont space, and contributions of CO_2 and HCO_3^- to steady-state photosynthesis, based on data in Tansik et al. (2015) and Tansik et al (2017). However, the presence of 20 mol · m^{-3} TRIS in the medium would have inhibited HCO_3^- use dependent on acid zones on the coral surface (Walker et al. 1980, Price and Badger 1985).

The pH in the compartments (exhabitant and inhabitant compartments) in the symbiotic corals was estimated by Venn et al. (2009), Barot et al. (2015), and Venn et al. (2019; see also Brownlee et al. 2009, Davy et al. 2012), using the fluorescence of the pH-sensitive SNARF dye. The pH in the cytosol of the various cells of the cnidarians, and in the photobionts, was in the range 7.0-8.0, with a lower pH in the peri-photobiont compartment.

These estimates involved fluorescent pH dyes. Laurent et al. (2014) examined the effects of decreased seawater pH on intracellular pH (again using SNARF fluorescence) in symbiont-free endoderm cells and in cells containing Symbiodiniaceae, showing that recovery from the initial decrease in intracellular pH probably involved a plasmalemma Na^+-H^+ antiporter. An additional possibility for pH regulation in the peri-photobiont space is the expression of a P-type H^+ ATPase in the plasmalemma of symbiotic, but not free-living cells, Symbiodiniaceae cells (Mies et al. 2017a,b).

No such fluorescence techniques are available for measuring the intracellular concentration of components of the inorganic C system. Measurements that have been made refer to estimates of the time course of intracellular ^{14}C-inorganic C during incubation in ^{14}C-inorganic C (Furla et al. 2000) refer to the bulk intracellular compartments. Furla et al. (2000) found that the equilibrium intracellular:bulk medium ratio of inorganic C was 1.56 in the dark and 61.3 in the light, using the tissue concentration of inorganic C of 147 mol · m^{-3} in the light (Furla et al. 2000). With tissue pH in the range 7-8 (Venn et al. 2009), the CO_2 concentration is 2 - 20 mol · m^{-3} (table 5.1 of Raven 1984). At face value, such an accumulation of inorganic C in the light would be expected to obviate the need for CCMs in the photobionts. $K_{0.5}$ CO_2 cannot be measured in vitro for Form II RuBisCO from dinoflagellates (see above); if it is similar to other Form II RuBisCOs, i.e., 100-250 mmol · m^{-3} (Horken and Tabita 1999, Robinson et al. 2003), and assuming that there is just enough Rubisco in the cell to give a CO_2-saturated carboxylation rate equal to the inorganic C saturated rate of photosynthesis in vivo, then even the lower estimate of intracellular CO_2 (2 mol · m^{-3}) in the cnidarian tissue would be sufficient to saturate in vivo photosynthesis. It is unlikely that such an inorganic C accumulation by the (future) exhabitant would pre-date the origin of the incorporation of the inhabitant as a 'pre-adaptation to symbiosis' (see Raven 1993).

A puzzling feature of the data of Furla et al (2000) is that the $t_{½}$ for ^{14}C inorganic C equilibration between the medium and the intracellular volume was 72 min in the dark and 106 min in the light. It is expected that the equilibration of ^{14}C would be the same within ~0.2% as that of bulk stable (^{12}C and ^{13}C) C isotopes (pp.118-122 of Zeebe and Wolf-Gladrow 2001, Zeebe 2011). As was mentioned under free-living dinoflagellates, a similarly long equilibration time was found by Berman-Frank and Erez (1996) for *Peridinium gatunense.*

Finally, the scyphozoan medusa *Cassiopeia* sp. has photobionts from the Symbiodiniaceae (Rädecker et al. 2017). Glucose enrichment increases respiratory O_2 uptake by the holobiont and photosynthesis (as O_2 evolution) by the photobionts in hospite; the stimulation of O_2 production was interpreted by Rädecker et al. (2017) as increased CO_2 availability to the photobiont, implying that photobiont photosynthesises in the absence of glucose in CO_2-limited conditions.

Rhizaria: symbiotic Foraminifera (calcified) and Radiolaria (silicified)

Inorganic C influx and internal inorganic pools have been investigated for Foraminifera with Symbiodiniaceae inhabitants (ter Kuile and Erez 1987, 1988, ter Kuile et al. 1989). Two species of Foraminifera were examined: the perforate species *Amphistegina lobifera* accumulates dissolved inorganic C in the light, while the imperforate *Amphisoris hemprochii* does not accumulate a significant inorganic C pool (ter Kuile et al. 1989). The dissolved inorganic C in *Amphistegina lobifera* may constitute separate pools related to calcification and photosynthesis. There seem to be no relevant data for radiolarians.

Bivalve molluscs: tridacnid clams

Tridacnid clams gain different proportions of their organic carbon from photosynthesis by their photobionts as a function of species, habitat and size, with the remainder of the organic carbon coming from phagotrophy (Klumpp and Lucas 1994; cf. Fisher et al. 1985, Jantzen et al. 2008, Mies et al. 2012). Differing from the situation in cnidarians, the Symbiodiniaceae cells are extracellular in these bivalve gastropods, occurring in the tubular evaginations of the gut in the siphonal mantle (Norton et al. 1992, Armstrong et al. 2018, Ip et al. 2018). It is not clear whether any of the small fraction of Symbodiniaceae cells found in four other organs of *Tridacna squamosa* (Poo et al. 2020) can bring about net inorganic C assimilation under in situ light conditions. The inorganic carbon used by Symbiodiniaceae in excess of that supplied by exhabitant and inhabitant respiration, permitting net photosynthesis in the light, must enter through the gill (ctenidia) plasmalemma over which seawater passes and across the epithelium to the membrane bounding the haemolymph, before circulating in the haemolymph to the epithelium of the gut evagination, the z-tubules (zooxanthella tubules;

fig. 10a of Ip et al. 2018). Inorganic carbon then crosses the z-tubule epithelium into the lumen containing the Symbiodiniaceae cells (fig. 10b of Ip et al. 2018).

On the basis of the projected area of mantle over which photosynthetically active radiation absorption occurs, gross light-saturated photosynthesis is 33 μmole · m^{-2} · s^{-1} (fig. 3 of Jantzen et al. 2008). From data in figure 4 of Jantzen et al. (2008), assuming the vertical axis units are correct rather than those in the legend to fig. 4) the incident photon cost of gross photosynthesis is about 20 μmole photon per mole O_2. There seem to be no data on the ctenophore area per giant clam or per kg fresh mass of clam, so the ctenidial membrane area-based inorganic C entry rate cannot be calculated. Whatever the inorganic C flux, a novel dual domain carbonic anhydrase may be involved in the transport process (Yellowlees et al. 1993, Leggat et al. 2005, Ip et al. 2017, Koh et al. 2018).

The haemolymph pH of *Tridacna gigas* varies from 7.2 at night and 8.1 in the light (Fitt et al. 1995); haemolymph inorganic C is 1.2 mole · m^{-3} at night and 0.8-0.6 mole · m^{-3} in the light (Rees et al. 1993, Yellowlees et al. 1993, Fitt et al. 1995, Leggat et al. 2000). The haemolymph pH in the light is similar to that of bulk seawater, but inorganic C is only 30-40% that of seawater. This gives an inwardly directed concentration gradient (seawater to haemolymph) for CO_2 and for HCO_3^-, and hence an inwardly directed free energy gradient from seawater to haemolymph for CO_2. Whether there is also such a free energy gradient for HCO_3^- depends on the electrical potential difference between seawater and haemolymph; no such measurements seem to have been made.

To reach the Symbiodiniaceae cells the inorganic C in the haemolymph must cross the z-tubule epithelium into the lumen (fig. 10b of Ip et al. 2018). One component of the transport mechanism is a V-type H^+ ATPase (more commonly found in endomembranes) in the plasmalemma of the luminal side of the epithelium, secreting H^+ into the lumen (Ip et al. 2018). Inhibition of the V-type ATPase decreases the rate of Symbiodiniaceae inhabitant by 40% (Armstrong et al. 2018). Armstrong et al. (2018) and Ip et al. (2018) suggest that there is a HCO_3^- transporter in the luminal membrane working in parallel with H^+-ATPase.

There are no reports of the inorganic C concentration in the z-tubule lumen; if it is no higher than in the haemolymph in the light, the CO_2 concentration is insufficient to saturate Form II RuBisCO. Even if there is an excess of RuBisCO activity over that needed to account for CO_2-saturated photosynthesis in the tridacnid, the Symbiodiniaceae cells would require

operation of a CCM (see Raven and Beardall 2017). There is expression of a P-type H^+-pumping ATPase in the plasmalemma of Symbiodiniaceae symbiotic with *Tridacna*, and also in a coral where the photobiont is intracellular, but not when the Symbiodiniaceae speces is free-living (Mies et al. 2017a,b). it is not clear how, if at all, this relates to functioning of a CCM.

Symbiodiniaceae are also symbiotic with some species of the bivalve subfamily Fraginae (Li et al. 2018). Natural abundance studies of carbon isotopes suggest that photosynthesis by the inhabitants contributes to the organic carbon in the holobiont, but there is no mechanistic information on inorganic C acquisition (Li et al. 2018). The Symbiodiniaceae are also extracellular inhabitants of bivalves of some Fraginae (cockles; Li et al. 2018), although there seems to be no information on inorganic C accumulation.

Conclusions on inorganic C acquisition by symbioses with dinoflagellates as photobionts

Members of the marine dinoflagellate family Symbiodiniaceae (Form II RuBisCO) are photobionts in a range of marine phagotrophs: Foraminifera, Radiolaria, Ciliata, Cnidaria, Acoela (which have *Amphidinium* as an additional photobiont) and Mollusca. The dinoflagellates are intracellular in all but the Mollusca (bivalves) where the Symbiodiniaceae are extracellular within the metazoan body. Almost all the data on inorganic C acquisition in these symbioses is for Foraminifera, Cnidaria, and tridacnid Mollusca. The data on Foraminifera are confined to measurements of intracellular inorganic C in two species, in only one of which is inorganic C is accumulated, and it is not clear that the accumulated pool relates to photosynthesis rather than calcification. Work on corals particularly (Cnidaria) shows significant inorganic C accumulation in the light, possibly obviating the need for a CCM in the photobiont. By contrast, the photobionts in the medusa *Cassiopeia* (Cnidaria) and in tridacnid clams are inorganic C limited for photosynthesis and require operation of a CCM. The high area-based photosynthetic rate of corals means that inorganic C entry must involve HCO_3^-. As such, where associations have evolved between specific species of Symbiodiniaceae and either giant clams (e.g., Lee et al. 2015) or corals (e.g., LaJeunesse et al. 2018), these outcomes raise the intriguing notion that lessons learnt on inorganic carbon acquisition and utilisation for one model symbiosis may not easily apply to the other.

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Table 1: Origin of chloroplasts and mitochondria in chromerids and dinoflagellates, and their photosynthetic accessory pigments and the RuBisCO form.

Exhabitant Taxon	Photosynthesis Taxon	Accessory Pigments	Form of RuBisCO	Mitochondria
Chromera	Chromera (g)	Chl c, peridinin	Form II	Chromera
Dinophyceae	Dinophyceae (g)	Chl c, peridinin	Form II	Dinophyceae
Dinophyceae	Prasinophycee (g or k)	Chl b, carotenoids	Form IB(ch)	Dinophyceae
Dinophyceae	Cryptoophyta (g or k)	Chl c, phycobilins	Form ID	Dinophyceae
Dinophyceae	Haptophyta (g or k)	Chl c, 19'haxanoyl= Fucoxanthin	Form ID	Dinophyceae
Dinophyceae	Bacillariophyceae (g or k)	Chl c, Fucoxanthiin	Form ID	Dinophyceae Bacillariophyceae
Dinophyceae	Cyanobacteria	Phycobilins	Form IB(cy)	Dinophyceae,
Dinophyceae	Prasinophyceae (k)	Chl b, carotenoids	Form IB(ch)	Dinophyceae
Dinophyceae	Dictyochophyceae (k)*	Chl c, Fucoxanthin, 19'butanoylo-fucoxanthin 19'haxanoyo-lfucoxanthin	Form ID	Dinophyceae
Dinophyceae	Pelagophyceae (k)*	Chl c Fucoxanthin, 19'butanoylo-fucoxanthin,	Form ID	Dinophyceae

(g) = genomically integrated chloroplasts (organisms in the case of dinotoms)

(k) = Kleptoplastids

(cy) = Cyanobacterial Form IB Rubisco or, for extracellular cyanobacterial symbionts, Form IA Rubisco.

(ch) = Chlorophyte/streptophyte Form IB Rubisco.

*Present, on molecular genetic evidence, at very low abundances among a diversity of plastids.

Table 2: Number of membranes that inorganic C crosses between the external medium and the plasmalemma of the dinoflagellate inhabitant in symbioses with dinoflagellates as the inhabitant (endosymbiont).

Exhabitant	Location of inhabitant	Number of membranes between medium and inhabitant plasmalemma
Rhizaria: Foraminifera	Intracellular	2
Rhizaria: Acantharia	Intracellular	2
Rhizaria: Radioaria	Intracellular	2
Alveolata: Ciliata	Intracellular	2
Metazoa: Porifera	Intracellular	2
Metazoa: Cnidaria	Intracellular	4[1]
Metazoa: Acoela	Intracellular	2?
Matazoa: Mollusca	Extracellular	4[2]

[1]Inorganic C moves from the external seawater medium (rather than the coelenteron) into, across and out of the aboral ectoderm, through the mesogloea, into the aboral endoderm, then across the perisymbiont membrane (fig. 3B of Gattuso et al. 1999). The total of 4 membranes rests on the assumption that the inorganic carbon uses the transcellular rather than the pericellular pathway across the aboral ectoderm (Gattuso et al. 1999).

[2]Inorganic C moves into, across and out of the ctenidial epithelium into the haemolymph, then into, across and out of the epithelium of the Z-tubule which contains the inhabitant cells in extracellular solution (fig. 10 of Ip et al. 2018).