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RESEARCH PAPER

Diversity of CO_2-concentrating mechanisms and responses to CO_2 concentration in marine and freshwater diatoms

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Abstract

The presence of CO_2-concentrating mechanisms (CCMs) is believed to be one of the characteristics that allows diatoms to thrive in many environments and to be major contributors to global productivity. Here, the type of CCM and the responses to variable CO_2 concentration were studied in marine and freshwater diatoms. At 400 ppm, there was a large diversity in physiological and biochemical mechanisms among the species. While *Phaeodactylum tricornutum* mainly used HCO_3^-, *Thalassiosira pseudonana* mainly used CO_2. Carbonic anhydrase was an important component of the CCM in all species and C_4 metabolism was absent, even with *T. weissflogii*. For all species, at 20 000 ppm, the affinity for dissolved inorganic carbon was lower than at 400 ppm CO_2 and the reliance on CO_2 was higher. Despite the difference in availability of inorganic carbon in marine and fresh waters, there were only small differences in CCMs between species from the two environments, and *Navicula pelliculosa* behaved similarly when grown in the two environments. The results suggest that species-specific differences are great, and more important than environmental differences in determining the nature and effectiveness of the CCM in diatoms.

Key words: Bicarbonate use, carbonic anhydrase, C_4 photosynthesis, carbon dioxide-concentrating mechanism, CCM, diatoms, PEP carboxylase, pH-drift, photosynthesis, PPDK, Rubisco.

Introduction

Diatoms currently contribute about 50% of the phytoplankton primary production in the oceans and are found in all aquatic environments (Armbrust, 2009). They evolved about 250 to 190 Mya (Sorhannus, 2007; Medlin, 2016) and became the most abundant phytoplankton class approximately 100 Mya, replacing green algae and cyanobacteria, which had dominated the oceans until that time (Armbrust, 2009). Their success may be linked to the increase in oceanic sulphate concentration between the Paleozoic and the Mesozoic Eras (270 Mya), and their competitive ability to continue uptake of some key nutrients when sulphate concentrations are high (Prioretti *et al.*, 2016). However, many other adaptations allow diatoms to out-compete other phytoplankton groups in today's oceans. For instance, the diatom silica cell wall may discourage attack by grazing organisms, regulate sinking rate (Raven and Waite, 2004), and serve as a pH buffer (Ellwood and Hunter, 2000; Milligan and Morel, 2002; Falkowski *et al.*, 2004; Katz *et al.*, 2005). Because of their photophysiology, diatoms are favoured over other phytoplankton groups in environments with fluctuating light, as occurs in non-stratified water columns (Taddei *et al.*, 2016).

In the aquatic environment, various nutrients, including CO_2, can limit diatom growth. CO_2 concentration has a direct impact on photosynthesis, but also influences other metabolic

pathways such as glycolysis, the Krebs cycle, nitrogen metabolism, and lipid synthesis (Tsuzuki *et al.*, 1990; Levitan *et al.*, 2007; Chiu *et al.*, 2009; Mus *et al.*, 2013; Kustka *et al.*, 2014; Mekhalfi *et al.*, 2014*a*; Wang *et al.*, 2014). However, evolution has conferred diatoms with the ability to acquire and store nutrients, and particularly carbon dioxide (CO_2), efficiently and rapidly, which is an ecological advantage in variable environments (Falkowski *et al.*, 2003). In marine and fresh waters, CO_2 concentrations are often too low to saturate the ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), which has an affinity constant for CO_2 of between 23 and 68 µM in diatoms (Badger *et al.*, 1998; Young *et al.*, 2016). Rubisco catalyses the first reaction of the Calvin–Benson–Bassham cycle, where the carboxylation of ribulose-1,5-bisphosphate (RuBP) produces two molecules of 3-phosphoglycerate. However, when the CO_2 concentration is low, Rubisco performs an oxygenation reaction, leading to the production of 2-phosphoglycolate (Bowes *et al.*, 1971), which must be metabolized through the photorespiration (C2) pathway (Anderson, 1971; Ogren, 1984; Sharkey, 1988; Douce and Heldt, 2000). CO_2-concentrating mechanisms (CCMs) that concentrate CO_2 around Rubisco have evolved to minimize photorespiration and optimize carboxylation (Badger *et al.*, 1998). Diatoms adapted to low CO_2 concentration have active CCMs (Burkhardt *et al.*, 2001; Trimborn *et al.*, 2008, 2009; Hu and Gao, 2009; Wu *et al.*, 2010; Yang and Gao, 2012; Nakajima *et al.*, 2013; Clement *et al.*, 2016).

Two types of CCM can be recognized: a biophysical CCM based on transport of CO_2/HCO_3^- and a biochemical CCM based on C_4 photosynthesis or crassulacean acid metabolism (CAM) photosynthesis. Carbonic anhydrases (CAs) that interconvert CO_2 and water into carbonic acid that rapidly dissociates to form HCO_3^- and protons ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$) play a fundamental role in the biophysical CCMs of cyanobacteria, other eukaryotic algae (Price *et al.*, 1992; Giordano *et al.*, 2005; Badger *et al.*, 2006), and diatoms (Patel and Merrett, 1986; Nimer *et al.*, 1998; Harada *et al.*, 2005; McGinn and Morel, 2008*b*; Crawfurd *et al.*, 2011; Matsuda *et al.*, 2011; Tachibana *et al.*, 2011; Hopkinson *et al.*, 2013; Kustka *et al.*, 2014; Samukawa *et al.*, 2014Clement *et al.*, 2016). CAs are located in many compartments in diatoms (Samukawa *et al.*, 2014) and these locations, as well as their number, vary among species. For instance, in *Thalassiosira pseudonana*, of 13 putative CAs, two (δ and γ) are external (eCAs) and localized at the periplasmic space, while in *Phaeodactylum tricornutum*, which has nine genes encoding CAs , eCAs are absent (Tanaka *et al.*, 2005; Tachibana *et al.*, 2011; Samukawa *et al.*, 2014; Hopkinson *et al.*, 2016). This absence in *P. tricornutum* seems to be compensated for by the presence of CO_2/HCO_3^- transporters, one of which has been well characterized (Nakajima *et al.*, 2013).

Some aquatic plants have C_4 and CAM photosynthesis. In these macrophytes, as in C_4 or CAM terrestrial plants, the first carboxylating enzyme is phosphoenolpyruvate carboxylase, which converts phosphoenolpyruvate and HCO_3^- into oxaloacetate (Sage, 2004). This C_4 syndrome has been found in *Hydrilla verticillata*, *Egeria densa*, and *Ottelia alismoides* (Magnin *et al.*, 1997; Casati *et al.*, 2000; Zhang *et al.*, 2014), but is not based on two different types of cells (Reiskind and Bowes, 1991; Lara *et al.*, 2002) unlike most, although not all, terrestrial plants (Voznesenskaya *et al.*, 2001, 2002; Freitag and Stichler, 2002). Since C_4 metabolism can occur in a single cell, it is possible that it could occur in diatoms. So far, *T. weissflogii* is the sole diatom species identified where the presence of a C_4 or C_3–C_4 intermediate photosynthesis seems to occur (Reinfelder *et al.*, 2000, 2004; Roberts *et al.*, 2007). In contrast, in *T. pseudonana* and *P. tricornutum*, the results are rather contradictory. Although there is some evidence for C_4 photosynthesis in these species (Reinfelder *et al.*, 2004; McGinn and Morel, 2008*a*; Kustka *et al.*, 2014), there is a growing body of work on *T. pseudonana* (Roberts *et al.*, 2007; Raven, 2010; Tanaka *et al.*, 2014 Clement *et al.*, 2016) and *P. tricornutum* (Haimovich-Dayan *et al.*, 2013; Yang *et al.*, 2016) using different approaches that suggest they have C_3 metabolism.

The large heterogeneity of CO_2 concentration strategies within diatoms, including highly diverse values for their Rubisco catalytic constants (Young *et al.*, 2016) and different distributions and numbers of CAs (Samukawa *et al.*, 2014), may be linked to the environmental conditions in their habitats. Our aim in this study was to determine the types of CCMs in different diatoms and whether or not they differ in species from marine and fresh waters. We extended an experimental strategy previously used successfully on *T. pseudonana* (Clement *et al.*, 2016) to other diatoms that live in either oceans, fresh waters, or both. We analysed the photosynthetic performance and the activities of enzymes involved in carbon acquisition and carboxylation, including CAs, Rubisco, and PEPC, in response to low (400 ppm) vs high (20 000 ppm) CO_2 concentration.

Materials and methods

Culture conditions

Thalassiosira pseudonana Hasle & Heim., strain CCAP 1085/12 (equivalent to CCMP1335), *Phaeodactylum tricornutum*, strain RCC Pt1_8.6 (equivalent to CCMP2561), *T. weissflogii*, strain CCAP 1085/18, and *Navicula pelliculosa*, strain CCAP 1050/9 were grown in artificial seawater supplemented with F/2+Si medium as described previously (Clement *et al.*, 2016). *Navicula pelliculosa* was also grown in a freshwater medium based on a modified artificial seawater plus F/2+Si medium in which NaCl and KCl were omitted and $NaH_2PO_4.2H_2O$ (0.036 mM) was replaced with KH_2PO_4 (0.036 mM). *Asterionella formosa*, isolated from Esthwaite Water in the English Lake District (strain BG1, isolated in Spring 2015), and *Aulacoseira granulata* var. *angustissima*, strain CCAP 1002/2, were grown in Diatom Medium as described previously (Mekhalfi *et al.*, 2012) and supplemented with metals (0.077 µM $ZnSO_4$, 0.041 µM $CuSO_4$, and 0.042 µM $CoCl_2$). Cultures were maintained at 16 °C with continuous illumination at 50 µmol photon m^{-2} s^{-1} photosynthetically active radiation, and constantly shaken at 90 rpm and bubbled with either 400 ppm or 20 000 ppm CO_2 at a gas flow rate of 130 ml min^{-1} as described previously (Clement *et al.*, 2016).

pH-drift experiments

Diatom cells from the exponential phase of growth were collected by centrifugation at 3720 *g* at 16 °C for 10 min, washed two times with a different medium for marine or freshwater species (see below), and

then diluted in the same medium to give a final cell density of between 200 000 and 5 000 000 cells ml^{-1} depending on the species. The medium for marine species was artificial seawater (pH 8) as described by Clement *et al.*, (2016) but with 2.19 instead of 4.39 mM $CaCl_2$ to minimize calcite precipitation. The medium for *As. formosa* and *Au. granulata* contained 5.8 µM $MgSO_4$, 34.4 µM $CaCl_2$, 1.3 µM NaCl, and 2.4 µM KCl (pH 8). The medium for *N. pelliculosa* cultivated in freshwater contained 20.8 mM $MgSO_4$, 2.19 mM $CaCl_2$, 0.88 mM NaCl, and 0.36 mM KCl. All media were supplemented with either 0.4 or 2 mM $NaHCO_3$. The cells were transferred to triplicate 15 ml Falcon tubes and placed in an incubator at 16 °C and illuminated continuously by fluorescent tubes producing about 170 µmol photon m^{-2} s^{-1} photosynthetically active radiation, as described previously (Maberly *et al.*, 2015). pH was measured with a combination pH-electrode (PHEL-GS2, Labbox) and meter (pH201, Hanna Instruments) after 24 h, and then approximately after every 12 h until a maximum pH had been reached. Taking into account all species, the pH-drift experiments lasted between 5 and 47 h. The geometric mean of the maximum pH reached was calculated along with the standard deviation.

Kinetics of O_2 evolution

Rates of net photosynthesis were measured as oxygen evolution at pH 7 and pH 8, 16 °C, 200 µmol photon m^{-2} s^{-1}, and increasing concentrations of $NaHCO_3$ from 10 to 2000 µM as described previously (Clement *et al.*, 2016). Species grown in seawater were measured in artificial seawater containing 10 mM HEPES. For *As. formosa*, a medium mimicking the Esthwaite Water mineral composition was used (Smith *et al.*, 2002) containing 10 mM HEPES, 5.8 µM $MgSO_4$, 34.4 µM $CaCl_2$, 1.3 µM NaCl, and 2.4 µM KCl. For *N. pelliculosa* cultivated in freshwater, the medium contained 10 mM HEPES, 20.8 mM $MgSO_4$, 4.39 mM $CaCl_2$, 0.88 mM NaCl, and 0.36 mM KCl. For *Au. granulata*, the quantity of cells collected was insufficient to perform kinetic experiments. Chlorophyll was measured after extraction in 96% ethanol as previously described (Clement *et al.*, 2016). The kinetic responses at pH 7 and pH 8 were analysed with a model to distinguish between CO_2- and HCO_3^--dependent inorganic carbon uptake (Clement *et al.*, 2016).

Protein extraction

Soluble protein extracts were prepared as described previously (Erales *et al.*, 2008; Mekhalfi *et al.*, 2014*b*) in a 30-mM Tris, 4-mM EDTA buffer containing 0.1 mM NAD and 1 mM cysteine, pH 7.9. The concentration of soluble proteins in the crude extracts was assayed using the Bradford reagent from Bio-Rad (Hercules, CA, USA), using bovine serum albumin as a standard.

Enzyme activities

All enzyme activities were measured from proteins extracted from cells collected in the exponential phase. Rubisco and C_4 enzymes (PEPC, NAD-ME, and PPDK) were tested as described previously (Zhang *et al.*, 2014). CA was assayed spectrophotometrically as described previously (Clement *et al.*, 2016). For *Au. granulata*, the quantity of cells was insufficient to measure enzyme activities.

Results

Inorganic carbon uptake capacity

The ability to remove inorganic carbon was measured in diatom cells grown at 400 ppm CO_2 in pH-drift experiments. The maximal pH values at alkalinities of 0.4 and 2 mEq l^{-1} are shown in Table 1 together with the corresponding calculated concentrations of total inorganic carbon (C_T), CO_2, bicarbonate, and the quotient C_T/Alk, which represents the proportion of available carbon left at the end of the drift (Maberly and Spence, 1983). There was a diversity in the ability to extract inorganic carbon among species: *Au. granulata* was the least effective and possibly restricted to CO_2 since the minimum CO_2 concentration it could produce was about 0.2 µM, while *N. pelliculosa* and *P. tricornutum* were the most effective species with very low final concentrations of CO_2 (Table 1). Generally, the two freshwater species were less effective than the marine species at alkalinities of both 0.4 and 2.0 mEq l^{-1} in terms of C_T/Alk, and even more so in terms of final concentrations of CO_2 or HCO_3^-. At both alkalinities, *N. pelliculosa* extracted more inorganic carbon in seawater than in freshwater. The two species of *Thalassiosira* had very similar and relatively low carbon extraction abilities

Table 1. *Results from pH-drift experiments at two alkalinities (Alk). Values are the maximal pH and the corresponding minimal concentration of total inorganic carbon (C_T), and CO_2, HCO_3^- and the quotient of C_T/alkalinity. Values are the mean of triplicate experiments with the standard deviation in parentheses. FW, freshwater; SW, seawater.*

Environment	Species	Max pH	C_T (µM)	CO_2 (nM)	HCO_3^- (µM)	C_T/Alk
Alkalinity 0.4 mEq l^{-1}						
FW	*As. formosa*	9.92 (0.12)	258 (22)	44 (1)	167 (31)	0.65 (0.06)
FW	*Au. granulata*	9.49 (0.02)	328 (2)	193 (25)	274 (4)	0.82 (0.01)
FW	*N. pelliculosa*	10.22 (0.01)	200 (2)	13 (3)	97 (2)	0.50 (0.01)
SW	*N. pelliculosa*	10.19 (0.04)	138 (7)	1 (0.7)	10 (1)	0.35 (0.02)
SW	*P. tricornutum*	10.03 (0.03)	161 (5)	1 (0.01)	17 (2)	0.40 (0.01)
SW	*T. pseudonana*	9.59 (0.03)	208 (3)	11 (0.7)	50 (3)	0.52 (0.01)
SW	*T. weissflogii*	9.67 (0.02)	200 (1)	8 (0.3)	41 (1)	0.50 (0.00)
Alkalinity 2.0 mEq l^{-1}						
FW	*As. formosa*	10.49 (0.01)	1095 (7)	26 (1)	370 (9)	0.55 (0.00)
FW	*Au. granulata*	9.93 (0.03)	1441 (19)	237 (25)	931 (34)	0.72 (0.01)
FW	*N. pelliculosa*	10.45 (0.02)	1119 (12)	31 (3)	402 (17)	0.56 (0.01)
SW	*N. pelliculosa*	10.33 (0.07)	930 (18)	2 (0.7)	50 (8)	0.47 (0.01)
SW	*P. tricornutum*	10.34 (0.01)	930 (2)	2 (0.1)	50 (1)	0.47 (0.00)
SW	*T. pseudonana*	9.99 (0.02)	1013 (3)	10 (0.7)	113 (4)	0.51 (0.00)
SW	*T. weissflogii*	9.99 (0.01)	1013 (1)	10 (0.3)	113 (1)	0.51 (0.00)

while *N. pelliculosa* and *P. tricornutum* were the most effective at removing inorganic carbon. The alkalinity of the test medium did not have a large effect on the final concentration of CO_2 at the end of a drift.

Carbon uptake kinetics

The maximal rates of net photosynthesis (V_{net}^{max}) in diatoms grown at 400 ppm CO_2 measured at pH 7 were similar to those obtained at pH 8 for all species except for *As. formosa* where V_{net}^{max} was higher at pH 7 than at pH 8 ($P<0.05$; Table 2). In contrast, in cells grown at 20 000 ppm, rates at pH 7 were greater than at pH 8 for *As. formosa* ($P<0.001$), *P. tricornutum* ($P<0.05$), and *T. pseudonana* ($P<0.001$). When cells were tested at pH 7, V_{net}^{max} was not different at 400 vs 20 000 ppm for *T. weisflogii*, but was greater at 20 000 ppm for *As. formosa* ($P<0.001$), *N. pelliculosa* in freshwater ($P<0.05$), *P. tricornutum* ($P<0.05$), and *T. pseudonana* ($P<0.001$). In *N. pelliculosa* in seawater the rate was greater at 400 than at 20 000 ppm. When cells were tested at pH 8, V_{net}^{max} rates were greater at 20 000 ppm for *As. formosa* ($P<0.01$), and greater at 400 ppm for *N. pelliculosa* in seawater ($P < 0.05$) and *T. pseudonana* ($P<0.05$).

The half-saturation constants ($K_{1/2}$) for dissolved inorganic carbon (DIC) at pH 7 compared to pH 8 were lower or significantly different for all species and growth conditions except for *N. pelliculosa* in freshwater and *T. pseudonana* at high CO_2 (Table 2). Moreover, at 20 000 ppm, the $K_{1/2}$ values were higher than those at 400 ppm at both pH 7 and 8, in agreement with CCM induction at low CO_2. In general, there were no differences in $K_{1/2}$ for DIC for marine and freshwater species, although the two species of *Thalassiosira* had the lowest $K_{1/2}$ at pH 7 and 400 ppm of any species.

The different kinetic parameters measured at pH 7 and pH 8 are consistent with the different proportions of CO_2 and HCO_3^- present at these two pH values. We therefore used these data to calculate CO_2 and HCO_3^- uptake, as described previously (Clement *et al.*, 2016). The models fitted the data well, with average R^2 values for the biological replicates of between 0.92 and 0.99 (Table 3) apart from one previously published data set on *T. pseudonana* (Clement *et al.*, 2016). The modelled CO_2 compensation point was zero throughout, consistent with a very high affinity for this form of inorganic carbon, as was also indicated in the pH-drift experiments. All species grown at 400 ppm had a low $K_{1/2}$ for CO_2 (Table 3, Fig. 1). *Phaeodactylum tricornutum* grown at 400 ppm had a capacity to take up CO_2 that was about 10-times lower than all the other species but had a very high CO_2 affinity (Table 3). There appeared to be two groups of species with different abilities to use HCO_3^-: *N. pelliculosa* regardless of the growth medium and *P. tricornutum* with very high uptake capacities, and the other three species with approximately a five-times lower maximal rate. The CO_2 as well as the HCO_3^- kinetics for *N. pelliculosa* were similar in freshwater and seawater (Fig. 1B). At ambient concentrations of CO_2 (~16 µM for cells grown at 400 ppm and ~800 µM for cells grown at 20 000 ppm) and HCO_3^- (400 µM in freshwater and 2000 µM in seawater), *P. tricornutum* relied almost entirely on HCO_3^- while in contrast *As. formosa* and the two species of *Thalassiosira* relied on CO_2 more than HCO_3^- when these species were grown at 400 ppm (Fig. 1C).

There were substantial differences in CO_2 and HCO_3^- uptake kinetics in cells grown at 20 000 rather than 400 ppm. The $K_{1/2}$ value for CO_2 was 1.5- to 70-fold higher at 20 000 compared to 400 ppm; the $K_{1/2}$ value for *N. pelliculosa* grown in seawater changed least and the value for *P. tricornutum* changed most. When grown at 20 000 ppm, the form of inorganic carbon taken up by *P. tricornutum* switched remarkably from HCO_3^- to CO_2 (Fig. 1C, F). Regardless of the growth medium, *N. pelliculosa* used HCO_3^- and CO_2 to a similar extent at both 20 000 ppm (roughly 50% HCO_3^-), and 400 ppm (roughly 60% HCO_3^-). The uptake of CO_2 and HCO_3^- by *As. formosa* was also not strongly affected by the different CO_2 concentrations. As shown previously, *T. pseudonana* relied largely on CO_2 even at 400 ppm, but completely down-regulated its use of HCO_3^- at 20 000 ppm (Clement *et al.*, 2016). Under ambient conditions the cells grown at 20 000 ppm were almost completely saturated by the available CO_2 and HCO_3^- (Fig. 1F). However, at 400 ppm CO_2 (~16 µM) these cells would only be between 43% saturated in the case of *As. formosa* and about 80% saturated for the two species of *Thalassiosira* (Fig. 1F).

Table 2. *Kinetics of photosynthesis for five species of diatoms grown in marine or freshwater, at two CO_2 concentrations and measured at pH 7 or pH 8. Values are the mean of two or three biological replicates and three technical replicates with standard deviation in parentheses. $K_{1/2}$ DIC, the half-saturation concentration of dissolved inorganic carbon.*

		Maximum photosynthesis (µmol h^{-1} mg^{-1} Chl*a*)				$K_{1/2}$ DIC (µM)			
		pH 7		pH 8		pH 7		pH 8	
Environment	**Species**	**400**	**20 000**	**400**	**20 000**	**400**	**20 000**	**400**	**20 000**
FW	*As. formosa*	107 (1)	337 (9)	79 (16)	159 (11)	7 (2)	55 (30)	13 (2)	106 (44)
FW	*N. pelliculosa*	191 (6)	246 (33)	180 (25)	195 (30)	7 (0)	57 (37)	11 (1)	26 (1)
SW	*N. pelliculosa*	240 (63)	136 (5)	190 (15)	123 (15)	7 (1)	22 (7)	8 (3)	64 (11)
SW	*P. tricornutum*	150 (25)	235 (46)	159 (20)	199 (27)	10 (2)	35 (12)	15 (4)	279 (62)
SW	*T. pseudonana**	111 (3)	205 (17)	113 (3)	95 (8)	4 (1)	59 (23)	15 (2)	46 (19)
SW	*T. weissflogii*	127 (15)	122 (23)	111 (4)	106 (10)	3 (1)	20 (11)	7 (1)	105 (17)

* Data from (Clement *et al.*, 2016).

Table 3. *Modelled kinetics of CO_2-dependent and HCO_3^--dependent photosynthesis for five species of diatoms grown in marine or freshwater, at two CO_2 concentrations. Values are means with standard errors of the estimate in parentheses.*

Environment	Species	V_{net}^{max} (μmol O_2 h^{-1} mg^{-1} Chl*a*)		$K_{1/2}$ (μM)		CP (μM)		Slope (μmol O_2 h^{-1} mg^{-1} Chl*a* $μM^{-1}$)		R^2
		CO_2	HCO_3^-	CO_2	HCO_3^-	CO_2	HCO_3^-	CO_2	HCO_3^-	
400 ppm CO_2										
FW	*As. formosa*	71 (12)	34 (17)	2.6 (0.9)	ND	0.0 (0.0)	ND	30.0 (14.7)	ND	0.94
FW	*N. pelliculosa*	61 (27)	127 (39)	1.3 (.4)	4.0 (0.9)	0.0 (0.0)	3.8 (0.6)	44.8 (8.3)	31.4 (3.0)	0.97
SW	*N. pelliculosa*	107 (2)	158 (22)	2.6 (0.6)	ND	0.0 (0.0)	ND	42.9 (9.6)	ND	0.94
SW	*P. tricornutum*	7 (11)	151 (18)	0.1 (0.1)	12.0 (1.5)	0.0 (0.0)	1.3 (1.3)	129.4 (42.6)	12.7 (2.2)	0.93
SW	*T. pseudonana**	85 (9)	27 (9)	0.4 (0.1)	2.7 (0.4)	0.0 (0.0)	7.5 (0.7)	296 (38)	42 (6)	0.92
SW	*T. weissflogii*	101 (16)	16 (8)	0.1 (0.0)	ND	0.0 (0.0)	ND	1127 (142)	ND	0.94
20 000 ppm CO_2										
FW	*As. formosa*	310 (27)	75 (16)	37.6 (14.5)	ND	0.0 (0.0)	ND	8.8 (2.3)	ND	0.92
FW	*N. pelliculosa*	129 (1)	133 (39)	31.5 (5.9)	11.9 (7)	0.0 (0.0)	0.0 (0.0)	4.2 (0.7)	11.9 (7.0)	0.99
SW	*N. pelliculosa*	95 (38)	83 (28)	4.2 (1.5)	11.7 (1.2)	0.0 (0.0)	0.0 (0.0)	18.5 (5.2)	11.7 (1.2)	0.99
SW	*P. tricornutum*	201 (45)	51 (25)	7.4 (2.1)	ND	0.0 (0.0)	ND	28.9 (10.7)	ND	0.98
SW	*T. pseudonana**	202 (39)	0.0 (0.0)	3.8 (0.1)	-	0.0 (0.0)	-	53.0 (9.0)	-	0.60
SW	*T. weissflogii*	97 (9)	29 (24)	4.1 (3.3)	3.2 (5.6)	0.0 (0.0)	0.0 (0.0)	35.2 (24.6)	3.2 (5.6)	0.97

* Data from (Clement *et al.*, 2016).
V_{net}^{max}, the maximum rate of net photosynthesis; $K_{1/2}$, the half-saturation concentration; CP, the compensation point; Slope, the ratio of V_{net}^{max} to $K_{1/2}$; R^2, the average coefficient of determination. ND signifies concentrations that were too low to be assessed accurately and consequently slopes could also not be determined. '–' signifies no use of HCO_3^- and therefore no value is appropriate.

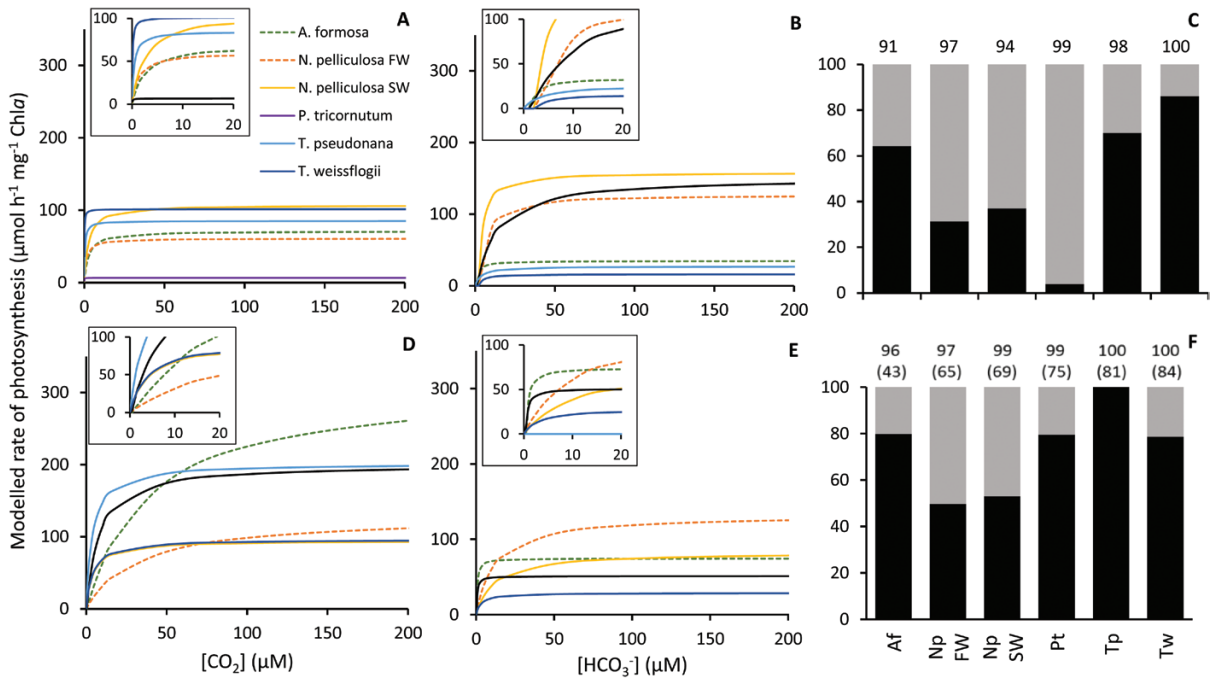

Fig 1. Modelled rates of net photosynthesis (μmol O_2 h^{-1} mg^{-1} Chl*a*). Rate as a function of CO_2 after growth at 400 ppm (A) or 20 000 ppm CO_2 (D), and as a function of HCO_3^- concentration after growth at 400 ppm (B) or 20 000 ppm CO_2 (E) are shown. The insets show the model outputs at low concentration. See (A) for the key to species; FW, freshwater; SW, seawater. The proportion (%) of CO_2 (black) and HCO_3^- (grey) contributing to net photosynthesis under ambient conditions (see text) for cells grown at 400 ppm (C) or 20 000 ppm CO_2 (F). The numbers given in (C) and (F) are the percent of carbon saturation under ambient conditions; in (F) the numbers in parentheses are the values when cells grown at 20 000 ppm were exposed to 400 ppm.

Enzyme activities

Rubisco, PEPC, NAD-ME, PPDK, and CA activities were measured with proteins extracted from the different diatoms cultured at either 400 ppm or 20 000 ppm CO_2 (Fig. 2). For all diatom species, Rubisco activity was between 1.3- and 1.8-fold higher and was significantly different (Student's *t*-test, $P<0.001$) in extracts from cells grown at 20 000 ppm vs 400 ppm (Fig. 2A).

Three diatoms, *T. weissflogii*, *N. pelliculosa* grown in freshwater, and *As. formosa*, had a significantly higher PEPC

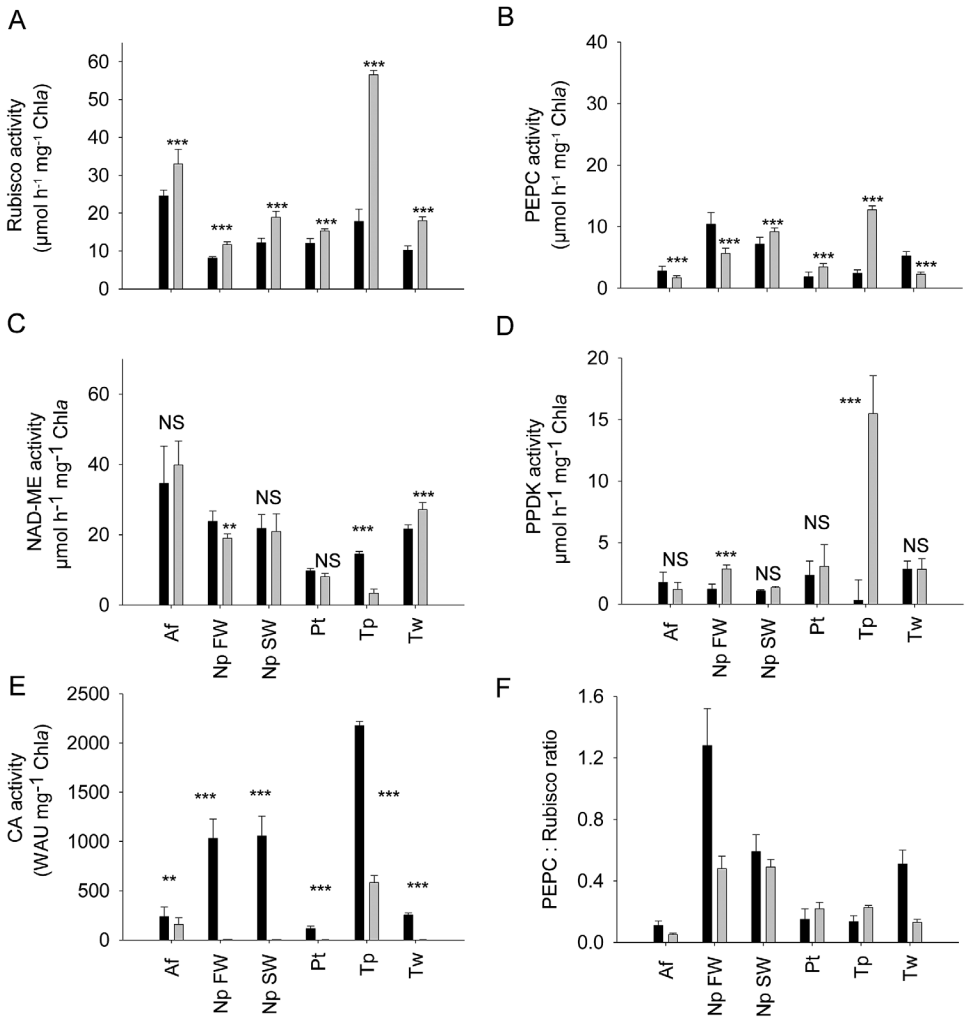

Fig. 2. Activities of Rubisco, C_4 enzymes, and carbonic anhydrase (CA) in diatoms grown at 400 ppm CO_2 (black bars) or 20 000 ppm CO_2 (grey bars). Rubisco activity (A), phosphoenolpyruvate carboxylase (PEPC) activity (B), NAD-malic enzyme (NAD-ME) (C), Pyruvate phosphate dikinase (PPDK) (D), and CA activity (E). The ratio of the activities of the two carboxylating enzymes, PEPC:Rubisco (F). Error bars represent the SD. $^{**}P<0.01$, $^{***}P<0.001$, NS stands for not significant (Student's *t*-test). For CA activity, WAU indicates Wilbur–Anderson Units. Af, *As. formosa*; Np FW, *N. pelliculosa* grown in freshwater; Np SW, *N. pelliculosa* grown in seawater; Pt, *P. tricornutum*; Tp, *T. pseudonana*; Tw, *T. weissflogii*.

activity (2.3-, 1.8-, and 1.6-fold, respectively; Student's *t*-test, $P<0.001$) at 400 ppm compared to 20 000 ppm (Fig. 2B). In contrast, *P. tricornutum*, *N. pelliculosa* grown in seawater, and *T. pseudonana* had significantly lower PEPC activity at 400 ppm (1.9-, 1.3-, and 5.3-fold, respectively; Student's *t*-test, $P<0.001$). At 400 ppm, the ratio of PEPC to Rubisco was below 0.6 in most diatoms except for *N. pelliculosa* grown in freshwater, where this ratio was 1.28 (Fig. 2F). NAD-ME activity did not significantly change in *As. formosa*, *P. tricornutum*, and *N. pelliculosa* in seawater regardless of CO_2 concentration (Fig. 2C). However, in *N. pelliculosa* in freshwater a higher activity was measured when grown at 400 ppm compared to 20 000 ppm (1.25-fold; Student's *t*-test, $P<0.01$). In contrast, a slightly higher activity (1.25-fold; Student's *t*-test, $P<0.01$) was measured in *T. weissflogii* at 20 000 ppm compared to 400 ppm. PPDK activity did not significantly change in *As. formosa*, *P. tricornutum*, *T. weissflogii*, and *N. pelliculosa* in seawater regardless of CO_2 concentration. However, in *N. pelliculosa* grown in freshwater, the activity of PPDK was 2.37-fold lower at 400 ppm compared to 20 000 ppm (Student's *t*-test, $P<0.001$) (Fig. 2D).

CA activity was higher in all diatom species grown at 400 ppm vs 20 000 ppm (Student's *t*-test, $P<0.001$ for all species except for *As. formosa*, $P<0.01$; Fig. 2E). CA activity was either not detected or very low at 20 000 ppm in *N. pelliculosa* from both environments, *P. tricornutum*, and *T. weissflogii*. CA activity at 400 ppm varied substantially among diatoms. In *As. formosa, P. tricornutum*, and *T. weissflogii* the CA activities were much lower than in *N. pelliculosa* (sea- and freshwater) and *T. pseudonana*.

Discussion

Diatoms inhabit a wide range of environments and the distribution of diatom taxa is strongly heterogeneous in the oceans (Malviya *et al.*, 2016) and in different freshwaters. In the oceans, the CO_2 concentration rarely falls substantially below 50% of the air-equilibrium concentration (~10 µM; Tortell, 2000; Hofmann *et al.*, 2011). In contrast, productive freshwaters are characterized by very variable CO_2, with concentrations substantially above or below air-equilibrium (Maberly, 1996). In addition, the HCO_3^- concentration in the oceans is

high and relatively constant, while it is very variable in different freshwaters (Raven and Maberly, 2004). Therefore diatoms from various habitats were studied to determine whether species from different environments have different capabilities to acquire inorganic carbon.

Physiological adaptation

There was a range of carbon-extraction abilities among the diatoms studied. Of the two freshwater species, *As. formosa* was more effective than *Au. granulata*, which agrees with a comparison made between natural populations of *As. formosa* and *Au. subarctica* (then known as *Melosira italica* subsp *subarctica*) (Talling, 1976). *Navicula pelliculosa* that had been grown in seawater was even more effective (Table 1), and similar to the freshwater *Fragilaria crotonensis*, also studied by Talling (1976). The final pH in drift experiments was greater at the higher alkalinity for each species, suggesting that pH *per se* was not limiting carbon uptake, at least at the lower alkalinity. The very low final CO_2 concentrations generated at the end of a drift (all less than 0.3 µM) suggest that all species, with the possible exception of *Au. granulata*, have a CCM. The final CO_2 concentrations at the end of a drift for the two freshwater species were slightly higher than for the marine species, but the overall amount of inorganic carbon available was similar.

In our study, the affinity ($K_{1/2}$) for DIC varied among diatoms and with CO_2 concentration. In all species, at 400 ppm the $K_{1/2}$ values for CO_2 were lower than the mean K_m for diatom Rubisco (Young *et al.*, 2016), further indicating that some form of CCM was present. In all the diatoms studied here, the affinities for DIC and CO_2 were lower for cells grown at 20 000 ppm vs 400 ppm, and this is also the case in all diatom species studied previously (Table 4), showing that the alteration of carbon kinetics in response to variable CO_2 is widespread in these algae. The $K_{1/2}$ values for CO_2 at 20 000 ppm were within the range reported for the K_m for diatom Rubisco (Young *et al.*, 2016), indicating that the CCM involved in CO_2 uptake had been completely down-regulated in most species. There is some indication of differences in carbon kinetics between different groups of diatoms (Table 4), but the large variability in values and the relatively few species studied means that more work is required to test this possibility. Despite a much higher HCO_3^- concentration in the medium, when grown at 400 ppm *As. formosa*, *T. weissflogii*, and *T. pseudonana* preferentially used CO_2. In contrast, *P. tricornutum* and *N. pelliculosa* grown in freshwater or seawater preferentially used HCO_3^-. In cells grown at 20 000 ppm, the capacity to use HCO_3^- or CO_2 was also heterogeneous among species, although CO_2 use became more important. These results are consistent with the diversity of CO_2/bicarbonate uptake already observed in other diatoms (Trimborn *et al.*, 2009). pH-drift experiments also confirmed a diversity in the ability to exploit DIC at low CO_2 (Talling, 1976).

Biochemical adaptation

In diatoms, CA is highly up-regulated at low CO_2 (Trimborn *et al.*, 2009; Crawfurd *et al.*, 2011; Hopkinson *et al.*, 2013, 2016; Kustka *et al.*, 2014; Clement *et al.*, 2016). Up-regulation of CA activity in low-CO_2 environments was observed for all the species. A 1.5-fold up-regulation of CA was observed in *As. formosa* grown at 400 vs 20 000 ppm, similar to the 4-fold up-regulation observed in *T. pseudonana* (Hopkinson *et al.*, 2013; Clement *et al.*, 2016). However, this is much less than the up-regulation observed in *P. tricornutum* and *T. weissflogii* (around 100-fold), and in *N. pelliculosa* grown in seawater and freshwater (240- and 223-fold, respectively). In *P. tricornutum*, where no external (periplasmic) CA has been found (Hopkinson *et al.*, 2013), CA activity was lower than in *T. pseudonana*. This result is consistent with data from the literature that indicate that in *P. tricornutum* bicarbonate transporters may play a major role in the CCM (Tachibana *et al.*, 2011; Nakajima *et al.*, 2013; Samukawa *et al.*, 2014) and with the preferential use of HCO_3^- reported here.

In C_4 metabolism, PEPC is expected to be the major carboxylating enzyme and its activity is expected to be higher than that of Rubisco; the ratio of PEPC:Rubisco therefore being greater than 1. In most species, except *N. pelliculosa* grown in freshwater, the activity of Rubisco was higher than the activity of PEPC, and therefore the ratio of PEPC:Rubisco was less than 1, irrespective of whether the cells were grown at low or high CO_2. These results are therefore not in favour of C_4 photosynthesis, including for *T. weissflogii*. Another criterion for C_4 metabolism is up-regulation, at low vs high CO_2, of enzymes involved in this type of photosynthesis, such as PEPC, PPDK that regenerates PEPC substrate, and malic enzymes that decarboxylate the C_4 compound to a C_3 compound producing CO_2 that can be fixed in the Calvin–Benson–Bassham cycle. In this study, NAD-ME, which preferentially uses NAD as a cofactor, was studied since NADP-ME has not been found in the genomes of the diatoms that have been sequenced so far (Kroth *et al.*, 2008). Indeed, the very low activity of NADP-ME that we measured (data not shown) is in agreement with the absence of this enzyme. Three diatoms had PEPC activity that was higher at 400 compared to 20 000 ppm, namely *T. weissflogii*, *As. formosa*, and *N. pelliculosa* grown in freshwater. However, in *T. weissflogii*, although PEPC increased at low CO_2, the ratio of PEPC:Rubisco was less than 1, PPDK was unaffected by CO_2, and NAD-ME activity was higher at high vs low CO_2. Overall, there is no strong evidence for C_4 metabolism for *T. weissflogii* under our experimental conditions. In *P. tricornutum*, PEPC also increased at low CO_2 but the value of the ratio of PEPC:Rubisco, and the unchanged activity of PPDK and NAD-ME, suggest that C_4 metabolism is absent in this diatom, in agreement with other recent work (Haimovich-Dayan *et al.*, 2013; Yang *et al.*, 2016). In this species, our study showed that CA activity increased at low CO_2 but that it was lower than in other species, in agreement with HCO_3^- transporters playing a major role (Hopkinson *et al.*, 2013). In *As. formosa*, where PEPC activity was also slightly higher in low vs high CO_2, NAD ME and PPDK were unaffected and the ratio of PEPC:Rubisco was much lower than 1, indicating that this species is unlikely to possess C_4 metabolism. In *N. pelliculosa*, when grown in freshwater, NAD-ME and PEPC activity were slightly higher at low vs high CO_2,

Table 4. *Kinetics of carbon uptake for marine and freshwater diatoms grown at different concentrations of CO_2. Cells grown and tested in freshwater media are indicated by (FW); the pH at which carbon (C) or oxygen exchange (O) was measured is given. $K_{1/2}$ DIC, the half-saturation concentration of dissolved inorganic carbon.*

Species	Class & sub-class*	[CO_2] (ppm)	pH	C or O_2	V_{max} (μmol h^{-1} mg^{-1} Chl*a*)	$K_{1/2}$ DIC (μM)	Reference
Cells grown at or below air-equilibrium							
Asterionella formosa (FW)	B-Fr	400	7	O_2	107	7	This study
Asterionella formosa (FW)	B-Fr	400	8	O_2	79	13	This study
Eucampia zodiacus	M-Bi	Air	8	C	414	323	Trimborn *et al.*, 2009
Navicula pelliculosa	B-Ba	Air	7.5	O_2	60	13	Colman and Rotatore, 1988
Navicula pelliculosa (FW)	B-Ba	400	7	O_2	191	7	This study
Navicula pelliculosa (FW)	B-Ba	400	8	O_2	180	11	This study
Navicula pelliculosa	B-Ba	400	7	O_2	240	7	This study
Navicula pelliculosa	B-Ba	400	8	O_2	190	8	This study
Nitzschia navis-varingica	B-Ba	59	8	C	290	301	Trimborn *et al.*, 2008
Nitzschia palea	B-Ba	400	8	O_2	210	85	Hu and Gao, 2009
Phaeodactylum tricornutum	B-	36	8	C	358	391	Burkhardt *et al.*, 2001
Phaeodactylum tricornutum	B-	180	8	C	394	574	Burkhardt *et al.*, 2001
Phaeodactylum tricornutum	B-	Air	8	C	340	743	Burkhardt *et al.*, 2001
Phaeodactylum tricornutum	B-	400	7	O_2	150	10	This study
Phaeodactylum tricornutum	B-	400	8	O_2	159	15	This study
Phaeodactylum tricornutum	B-	400	8.15	C	350	510	Wu *et al.*, 2010
Pseudo-nitzschia multiseries	B-Ba	243	8	C	369	223	Trimborn *et al.*, 2008
Skeletonema costatum	M-Th	36	8	C	316	17	Rost *et al.*, 2003
Skeletonema costatum	M-Th	180	8	C	374	138	Rost *et al.*, 2003
Skeletonema costatum	M-Th	Air	8	C	353	246	Rost *et al.*, 2003
Skeletonema costatum	M-Th	400	8	C	309	265	Trimborn *et al.*, 2009
Stellarima stellaris	C-Co	243	8	C	264	304	Trimborn *et al.*, 2009
Thalassionema nitzschioides	B-Fr	400	8	C	342	223	Trimborn *et al.*, 2009
Thalassiosira pseudonana	M-Th	50	7	O_2	156	13.3	Clement *et al.*, 2016
Thalassiosira pseudonana	M-Th	400	7	O_2	111	4	Clement *et al.*, 2016
Thalassiosira pseudonana	M-Th	400	8	O_2	113	15	Clement *et al.*, 2016
Thalassiosira pseudonana	M-Th	Air	8.2	O_2	180	15	Nakajima *et al.*, 2013
Thalassiosira pseudonana	M-Th	Air	8.2	O_2	227	101	Tanaka *et al.*, 2014
Thalassiosira pseudonana	M-Th	Air	8	C	484	513	Trimborn *et al.*, 2008
Thalassiosira pseudonana	M-Th	Air	8.2	C	535	276	Yang and Gao, 2012
Thalassiosira weissflogii	M-Th	36	8	C	188	104	Burkhardt *et al.*, 2001
Thalassiosira weissflogii	M-Th	180	8	C	251	196	Burkhardt *et al.*, 2001
Thalassiosira weissflogii	M-Th	Air	8	C	242	391	Burkhardt *et al.*, 2001
Thalassiosira weissflogii	M-Th	400	7	O_2	127	3	This study
Thalassiosira weissflogii	M-Th	400	8	O_2	111	7	This study
Cells grown above air-equilibrium							
Asterionella formosa (FW)	B-Fr	20 000	7	O_2	337	55	This study
Asterionella formosa (FW)	B-Fr	20 000	8	O_2	159	106	This study
Eucampia zodiacus	M-Bi	800	8	C	450	411	Trimborn *et al.*, 2009
Navicula pelliculosa (FW)	B-Ba	20 000	7	O_2	246	57	This study
Navicula pelliculosa (FW)	B-Ba	20 000	8	O_2	195	26	This study
Navicula pelliculosa	B-Ba	20 000	7	O_2	136	22	This study
Navicula pelliculosa	B-Ba	20 000	8	O_2	123	64	This study
Nitzschia navis-varingica	B-Ba	819	8	C	261	494	Trimborn *et al.*, 2008
Nitzschia palea	B-Ba	700	8	O_2	125	101	Hu and Gao, 2009
Phaeodactylum tricornutum	B-	1800	8	C	407	1174	Burkhardt *et al.*, 2001
Phaeodactylum tricornutum	B-	20 000	7	O_2	235	35	This study
Phaeodactylum tricornutum	B-	20 000	8	O_2	199	279	This study
Phaeodactylum tricornutum	B-	1000	8.15	C	370	610	Wu *et al.*, 2010
Pseudo-nitzschia multiseries	B-Ba	819	8	C	368	327	Trimborn *et al.*, 2008
Skeletonema costatum	M-Th	1800	8	C	347	505	Rost *et al.*, 2003
Skeletonema costatum	M-Th	800	8	C	371	441	Trimborn *et al.*, 2009
Stellarima stellaris	C-Co	819	8	C	258	572	Trimborn *et al.*, 2008
Thalassionema nitzschioides	B-Fr	800	8	C	364	379	Trimborn *et al.*, 2009

Table 4. *Continued*

Species	Class & sub-class*	[CO_2] (ppm)	pH	C or O_2	V_{max} (µmol h^{-1} mg^{-1} Chl*a*)	$K_{1/2}$ DIC (µM)	Reference
Thalassiosira pseudonana	M-Th	20 000	7	O_2	205	59	Clement *et al.*, 2016
Thalassiosira pseudonana	M-Th	20 000	8	O_2	95	46	Clement *et al.*, 2016)
Thalassiosira pseudonana	M-Th	50000	8.2	O_2	179	515	Nakajima *et al.*, 2013)
Thalassiosira pseudonana	M-Th	800	8	C	470	443	Trimborn *et al.*, 2008)
Thalassiosira pseudonana	M-Th	1000	8.2	C	497	402	Yang and Gao, 2012)
Thalassiosira weissflogii	M-Th	1800	8	C	260	509	Burkhardt *et al.*, 2001)
Thalassiosira weissflogii	M-Th	20 000	7	O_2	122	20	This study
Thalassiosira weissflogii	M-Th	20 000	8	O_2	106	105	This study

* Taxonomic position follows AlgaeBase (www.algaebase.org; searched on 9 January 2017). Class: B, Bacillariophyceae; C, Coscinodiscophyceae; M, Mediophyceae. Sub-class: Ba, Bacillariophycidae; Bi, Biddulphiophycidae; Co, Coscinodiscophycidae; Fr, Fragilariophycidae; Th, Thalassiosirophycidae. The precise taxonomic position of *Phaeodactylum tricornutum* is uncertain.

and the ratio of PEPC:Rubisco was slightly above 1; more work is therefore required to confirm if this diatom can use a C_4 metabolism. Lastly, in the well-studied diatom *T. pseudonana*, the presence of this photosynthesis type seems to be absent (Roberts *et al.*, 2007; Tanaka *et al.*, 2014; Clement *et al.*, 2016), although this has been controversial (McGinn and Morel, 2008*a*; Kustka *et al.*, 2014).

As shown previously in *T. pseudonana* and in all the species studied here, Rubisco activity decreased at low vs high CO_2 concentration (Clement *et al.*, 2016), consistent with down-regulating carboxylation capacity under carbon shortage. These results are in good agreement with data showing that Rubisco concentration was higher at 750 ppm than at 390 ppm CO_2 in *Emiliania huxleyi* and *T. pseudonana* (McCarthy *et al.*, 2012). It is, however, in contrast with data obtained for *T. weissflogii* comparing 750 to 396 and 182 ppm CO_2, where Rubisco concentration decreased at the highest CO_2 concentration (Losh *et al.*, 2013). While this study suggested that an increase in the rate of carbon fixation per unit of enzyme as external CO_2 increases would make it possible to reduce the Rubisco content while maintaining constant rates of carbon fixation and growth, our results suggest that while CCM is down-regulated at high CO_2, Rubisco activity is up-regulated.

To conclude, our work provides no compelling evidence that C_4 photosynthesis is present in any of the diatom species tested. In contrast, CA and active uptake of HCO_3^- are clearly widespread and important components of diatom CCMs There is large variation among diatoms in their reliance on CO_2 or HCO_3^- as an inorganic carbon source and in the type and effectiveness of their CCMs, but in the few species studied there are no clear differences between marine and freshwater species, despite the differences in carbon availability between the two environments.

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