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# Differential effects of ocean acidification on carbon acquisition in two bloom-forming dinoflagellate species

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Dinoflagellates represent a cosmopolitan group of phytoplankton with the ability to form harmful algal blooms. Featuring a Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) with very low $CO_2$ affinities, photosynthesis of this group may be particularly prone to carbon limitation and thus benefit from rising atmospheric $CO_2$ partial pressure ($pCO_2$) under ocean acidification (OA). Here, we investigated the consequences of OA on two bloom-forming dinoflagellate species, the calcareous *Scrippsiella trochoidea* and the toxic *Alexandrium tamarense*. Using dilute batch incubations, we assessed growth characteristics over a range of $pCO_2$ (i.e. 180–1200 µatm). To understand the underlying physiology, several aspects of inorganic carbon acquisition were investigated by membrane-inlet mass spectrometry. Our results show that both species kept growth rates constant over the tested $pCO_2$ range, but we observed a number of species-specific responses. For instance, biomass production and cell size decreased in *S. trochoidea*, while *A. tamarense* was not responsive to OA in these measures. In terms of oxygen fluxes, rates of photosynthesis and respiration remained unaltered in *S. trochoidea* whereas respiration increased in *A. tamarense* under OA. Both species featured efficient carbon concentrating mechanisms (CCMs) with a $CO_2$-dependent contribution of $HCO_3^-$ uptake. In *S. trochoidea*, the CCM was further facilitated by exceptionally high and $CO_2$-independent carbonic anhydrase activity. Comparing both species, a general trade-off between maximum rates of photosynthesis and respective affinities is indicated. In conclusion, our results demonstrate effective CCMs in both species, yet very different strategies to adjust their carbon acquisition. This regulation in CCMs enables both species to maintain growth over a wide range of ecologically relevant $pCO_2$.

*Abbreviations* – CA, carbonic anhydrase; CCM, carbon concentrating mechanism; Chl *a*, chlorophyll *a*; $C_i$, inorganic carbon; $CO_3^{2-}$, carbonate ion; DBS, dextran-bound sulphonamide; DIC, dissolved inorganic carbon; eCA, extracellular carbonic anhydrase; $HCO_3^-$, bicarbonate; HEPES, 4-(2-hydroxylethyl)-1-piperazine-ethanesulfonic acid; $K_{1/2}$, half-saturation concentration; MIMS, membrane-inlet mass spectrometry; OA, ocean acidification; $pCO_2$, atmospheric $CO_2$ partial pressure; PFD, photon flux density; PIC, particulate inorganic carbon; POC, particulate organic carbon; PON, particulate organic nitrogen; PST, paralytic shellfish poisoning toxins; RubisCO, Ribulose-1,5-bisphosphate carboxylase/oxygenase; TA, total alkalinity.

## Introduction

Since the Industrial Revolution, alterations in fossil fuel combustion and land-use have caused atmospheric $CO_2$ partial pressure ($pCO_2$) to increase from approximately 280 toward approximately 395 µatm at present-day, and is predicted to reach values of approximately 900 µatm by the end of the 21st century (IPCC 2007). Regarding the oceans, elevated $pCO_2$ causes an increase in $CO_2$ and bicarbonate ($HCO_3^-$) concentrations, while carbonate ion concentrations ($CO_3^{2-}$) decrease. These changes in the speciation of dissolved inorganic carbon (DIC) result in lowered pH values, a phenomenon also known as ocean acidification (OA; Wolf-Gladrow et al. 1999, Caldeira and Wickett 2003). OA and associated changes in the carbonate chemistry have been shown to impact marine organisms in many ways (Fabry et al. 2008). Especially for phytoplankton, being the base of the marine food web and the driver of the biological carbon pumps, such changes may have far reaching consequences (Falkowski et al. 1998, Doney et al. 2009).

Phytoplankton take up inorganic carbon and fix $CO_2$ into organic compounds by Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). This enzyme generally features low affinities for $CO_2$, and a competing reaction with $O_2$ further reduces its overall efficiency (Badger et al. 1998). To overcome these catalytic limitations imposed by RubisCO, phytoplankton developed so-called carbon concentrating mechanisms (CCMs). Common features of a CCM include active uptake of $CO_2$ and $HCO_3^-$ as well as means to reduce the $CO_2$ leakage (Giordano et al 2005, Rost et al. 2006). CCMs may further involve carbonic anhydrase (CA), an enzyme which accelerates the otherwise slow interconversion between $CO_2$ and $HCO_3^-$. The mode and cost of CCMs will to a great extent determine the sensitivity of phytoplankton toward OA (Rost et al. 2008, Reinfelder 2011).

The functioning of CCMs has been intensively studied in various phytoplankton species. In diatoms, CCMs were generally downregulated with increasing $pCO_2$ as reflected by lowered photosynthetic affinities for $CO_2$ and DIC (Burkhardt et al. 2001, Trimborn et al. 2008). Often, these changes were also accompanied by lowered contribution of $HCO_3^-$ uptake or decreased activities of extracellular CA (eCA). In other taxa such as the coccolithophore *Emiliania huxleyi* or the cyanobacterium *Trichodesmium*, affinities for $CO_2$ and DIC were also downregulated under OA, yet eCA activity did not seem to play a role in the functioning of their CCMs (Rost et al. 2003, Kranz et al. 2009). These different modes of CCMs and their regulation with $pCO_2$ have increased our understanding about species-specific responses toward OA in diatoms, coccolithophores and cyanobacteria. Little is yet known about other taxa, such as dinoflagellates.

Earlier work suggested severe $CO_2$ limitation in photosynthesis of dinoflagellates (Colman et al. 2002, Dason et al. 2004). This was attributed to their type II RubisCO, which has the lowest affinity for $CO_2$ of all eukaryotic phytoplankton (Morse et al. 1995, Badger et al. 1998), as well as limited ability to use $HCO_3^-$. Recent studies have, however, demonstrated high $HCO_3^-$ uptake rates in the dinoflagellates species *Ceratium lineatum*, *Heterocapsa triquetra*, *Prorocentrum minimum* (Rost et al. 2006, Fu et al. 2008) and *Protoceratium reticulatum* (Ratti and Giordano 2007), indicating rather efficient modes of CCMs that may make them relatively independent from changes in $CO_2$ availability. In some dinoflagellates, CCMs have been shown to respond to changes in carbonate chemistry, e.g. by lowered photosynthetic affinities for $CO_2$ and DIC (Rost et al. 2006, Ratti and Giordano 2007), or by downregulation of CA transcripts (Van de Waal et al. 2013) with increasing $pCO_2$. Such apparent differences in the regulation of CCMs may explain the observed variability in responses to OA in growth and primary production of different dinoflagellate species (Fu et al. 2007, 2010) or strains (Brading et al. 2011, 2013).

To improve our understanding about growth responses and the functioning of CCMs in dinoflagellates under OA, this study investigated the eco-physiology of two distinct dinoflagellate species, the calcareous *Scrippsiella trochoidea* and the toxic *Alexandrium tamarense* over a range of $pCO_2$. Both are bloom-forming species that co-occur in the North Sea (Fistarol et al. 2004, McCollin et al. 2011). As one has the potential to calcify and the other is a potent toxin producer, different ecological strategies can be expected, which may also be reflected in the functioning of their CCM. Hence, measurements on growth and biomass production were accompanied by measurements on inorganic carbon fluxes and CA activities using membrane-inlet mass spectrometry (MIMS).

## Materials and methods

### Species and growth conditions

*Scrippsiella trochoidea* GeoB267 (culture collection of the University of Bremen) and *Alexandrium tamarense* Alex5 (Tillmann et al. 2009), both isolates from the North Sea, were cultured at 15°C in 0.2 µm filtered North Sea water (salinity 34). Vitamins and trace metals were added according to f/2 medium (Guillard and Ryther 1962), except for $FeCl_3$ (1.9 µmol $l^{-1}$), $H_2SeO_3$ (10 nmol $l^{-1}$) and $NiCl_2$ (6.3 nmol $l^{-1}$). Nitrate and

phosphate were added to final concentrations of 100 and 6.25 µmol $l^{-1}$, respectively. Culture medium was pre-aerated with air containing $pCO_2$ of 180 µatm (Last Glacial Maximum), 380 µatm (present-day), 800 µatm and 1200 µatm (scenarios of the year 2100 and beyond). These concentrations were obtained by mixing $CO_2$-free air (<0.1 µatm $pCO_2$; Domnick Hunter, Willich, Germany) with pure $CO_2$ (Air Liquide Deutschland, Düsseldorf, Germany) using mass flow controllers (CGM 2000 MCZ Umwelttechnik, Bad Nauheim, Germany). $CO_2$ concentrations were regularly verified by a non-dispersive infrared analyzer system (LI6252, LI-COR Biosciences, Bad Homburg, Germany).

Cultures were grown in 2.4 l borosilicate bottles and placed on a roller table to allow homogenous mixing. Light was provided by OSRAM daylight tubes (18 W/965 Biolux) at a light : dark cycle of 16:8 h. Light was adjusted to an incident photon flux density (PFD) of 250 ± 25 µmol photons $m^{-2}$ $s^{-1}$ using a spherical micro quantum sensor (Walz, Effeltrich, Germany). Prior to the onset of the experiments, cells were acclimated to the respective $CO_2$ concentrations for at least 14 days. To ensure dilute batch conditions with minor changes in carbonate chemistry, cultures were diluted about once a week and population densities were kept <400 cells $ml^{-1}$. Experiments were run in triplicates (n = 3) over at least 5 days.

## Sampling and analyses

Samples were always taken 5–7 h after the start of the light period. Every other day, pH was measured with a 2-point calibrated WTW pH meter 3110 (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). Samples for total alkalinity (TA) were analyzed by a fully automated titration system (SI Analytics, Mainz, Germany) with a mean accuracy of 13 µmol $l^{-1}$. DIC samples were analyzed in a QuAAtro high performance microflow analyzer (Seal, Mequon, WI) with a mean accuracy of 8 µmol $l^{-1}$. Changes in TA and DIC over the course of the incubations were <2 and <3.4%, respectively. Owing to the decreasing buffer capacity with increasing $pCO_2$ (Egleston et al. 2010), DIC consumption caused higher variability in pH and $pCO_2$ in the high $CO_2$ treatments (Table 1). Carbonate chemistry was calculated with CO2sys (Pierrot et al. 2006) using $pH_{NBS}$ (National Bureau of Standards) and TA of each incubation. Equilibrium constants of Mehrbach et al. (1973), refitted by Dickson and Millero (1987) were chosen.

To determine population densities, 20–60 ml culture suspension was fixed with Lugol's solution (2% final concentration). Each day, triplicate cell counts were performed with an Axiovert 40C inverted microscope (Carl Zeiss MicroImaging GmbH, Hamburg, Germany). Specific growth rates ($\mu$) were calculated by an exponential fit through cell counts over at least 4 days for each biological replicate (n = 3).

**Table 1.** Carbonate chemistry for the different $CO_2$ treatments. Values for TA, DIC and pH indicate the mean of triplicate incubations (n = 3; ± SD). $pCO_2$ was calculated based on pH and TA of each incubation, using equilibrium constants by Mehrbach et al. (1973), refitted by Dickson and Millero (1987).

| $CO_2$ treatment | TA (µmol $l^{-1}$) | DIC (µmol $l^{-1}$) | $pH_{NBS}$ | $pCO_2$ (µatm) |
|---|---|---|---|---|
| *S. trochoidea* | | | | |
| 180 | 2386 ± 1 | 1972 ± 16 | 8.45 ± 0.01 | 180 ± 6 |
| 380 | 2388 ± 2 | 2096 ± 10 | 8.21 ± 0.02 | 358 ± 15 |
| 800 | 2385 ± 1 | 2223 ± 11 | 7.91 ± 0.03 | 785 ± 55 |
| 1200 | 2386 ± 4 | 2268 ± 18 | 7.77 ± 0.04 | 1133 ± 97 |
| *A. tamarense* | | | | |
| 180 | 2434 ± 3 | 1992 ± 33 | 8.50 ± 0.06 | 162 ± 24 |
| 380 | 2439 ± 1 | 2117 ± 41 | 8.27 ± 0.07 | 315 ± 57 |
| 800 | 2434 ± 2 | 2245 ± 37 | 7.97 ± 0.10 | 706 ± 154 |
| 1200 | 2418 ± 1 | 2283 ± 34 | 7.83 ± 0.12 | 995 ± 248 |

At the end of each experiment, samples were taken to assess particulate organic carbon and nitrogen (POC and PON), particulate inorganic carbon (PIC, as difference between total particulate carbon and POC), as well as chlorophyll *a* (Chl *a*). For analyses of POC and PON, 300–400 ml culture suspension was filtered in duplicate on pre-combusted GF/F filters (500°C, 6 h). Prior to POC measurements, 200 ml of HCl (0.1 mol $l^{-1}$) was added to the filters to remove all PIC, and filters were dried overnight. Filters were wrapped in tin foil cups and analyzed by an ANCA-SL 20–20 mass spectrometer (SerCon Ltd., Crewe, UK). To determine Chl *a*, 100–200 ml culture suspension was filtered in duplicate on cellulose-nitrate filters (Whatman, Maidstone, UK), rapidly frozen in liquid nitrogen and subsequently stored at −80°C. Extraction and fluorometric determination of Chl *a* were done according to Knap et al. (1996), using a TD-700 Fluorometer (Turner Designs, Sunnyvale, CA).

## Oxygen and inorganic carbon flux measurements

$O_2$ and $CO_2$ fluxes were measured by means of MIMS (Isoprime, GV Instruments, Manchester, UK) to determine photosynthetic $O_2$ evolution and respiratory $O_2$ uptake, as well as $CO_2$ and $HCO_3^-$ fluxes. Net $O_2$ fluxes were converted to inorganic carbon ($C_i$) fluxes by applying a photosynthetic quotient of 1.4 (as nitrate was the only nitrogen source in the growth medium) and a respiratory quotient of 1.0 (Williams and Robertson 1991). The applied approach by Badger et al. (1994) depends on a chemical disequilibrium between $CO_2$

and $HCO_3^-$, which is induced by photosynthetic $C_i$ uptake in the absence of eCA activity. $O_2$ and $CO_2$ fluxes were measured simultaneously during steady-state photosynthesis in consecutive light–dark intervals with increasing amounts of DIC. Maximum rates ($V_{max}$) and half-saturation concentrations ($K_{1/2}$) for respective $C_i$ species ($CO_2$ and $HCO_3^-$) and DIC were determined by applying a Michaelis–Menten fit. Negative estimates of $HCO_3^-$ concentrations, which were occasionally calculated for the lowest DIC concentrations, were omitted from the Michaelis–Menten fit. Measurements were performed in a 4-(2-hydroxylethyl)-1-piperazine-ethanesulfonic acid (HEPES, 50 mmol $l^{-1}$) buffer in f/2 medium with a pH of $8.0 \pm 0.1$ at $15 \pm 0.3^{\circ}C$. The applied pH in the MIMS assay represents an intermediate value of the pH values of the acclimations. Provided that these differences in pH have minor effects on $C_i$ uptake kinetics, rates in the assays are also representative for the acclimation. For more details on the method see Badger et al. (1994) and Rost et al. (2007).

Prior to the experimental series, the shape and speed of the stirrer in the MIMS-cuvette were tested on both species to eliminate biases from mechanical and physiological stress for the dinoflagellate species. In test runs, photosynthetic $O_2$ and respiration evolution was measured in intervals for about 1 h, confirming that rates remained unaffected over the duration of the assay. Light and dark intervals were adjusted to 4.5 and 3.5 min, respectively, to allow the $CO_2$ and $O_2$ traces to reach steady-state conditions (i.e. a linear slope; see Rost et al. 2006). The light intensity in the cuvette was set to the light intensity of the experiments (tested with the same light meter) with very comparable light spectra as similar daylight tubes have been used. Prior to the measurements, acclimated dinoflagellate cells were concentrated by gentle vacuum filtration (<200 mbar) over a 10 µm membrane filter (Millipore, Billerica, MA). Culture medium was exchanged with DIC-free assay medium and 8 ml of this concentrated cell suspension was transferred into the MIMS cuvette. During the first dark phase, membrane-impermeable dextran-bound sulphonamide (DBS; Synthelec AB, Lund, Sweden) was added to a final concentration of 50 µmol to inhibit any potential eCA activity. In order to normalize rates, duplicate Chl *a* samples were taken after each measurement.

### Extracellular carbonic anhydrase activities

The determination of eCA activity was monitored by the $^{18}O$ depletion rate of doubly labeled $^{13}C^{18}O_2$ in sea water via alternating hydration and dehydration steps (Silverman 1982). As CA catalyzes the interconversion between $HCO_3^-$ and $CO_2$, it concomitantly enhances the exchange of $^{18}O$ in $^{13}C^{18}O^{18}O$ (m/z = 49) with $^{16}O$ from water molecules, forming $^{13}C^{18}O^{16}O$ (m/z = 47) and subsequently $^{13}C^{16}O^{16}O$ (m/z = 45). In the dark, $NaH^{13}C^{18}O_3$ label was injected into the cuvette containing 8 ml HEPES-buffered culture medium with a pH of $8.0 \pm 0.1$ at $15 \pm 0.3^{\circ}C$. After recording the steady-state depletion in $^{18}O$ enrichment for approximately 8 min ($S_1$), 400 µl of the concentrated cell suspension was injected, and the $^{18}O$ depletion was followed for another 10 min ($S_2$). Units of eCA activity (U) were calculated using the catalyzed and non-catalyzed rates $S_2$ and $S_1$, respectively, and subsequently normalized to Chl *a* (Badger and Price 1989). As a consequence, U corresponds to the enhancement in the interconversion between $CO_2$ and $HCO_3^-$, expressed as % µg Chl $a^{-1}$. For more details on the method see Palmqvist et al. (1994) and Rost et al. (2007).

### Statistics

Normality of data was confirmed using the Shapiro-Wilk test. Variables were log-transformed if this improved the homogeneity of variances, as tested by Levene's test. Significant differences between treatments were tested using one way ANOVA, followed by post hoc comparison of the means using Tukey's HSD ($\alpha = 0.05$; Quinn and Keough 2002); significant differences between species, i.e. comparing the respective treatments, were tested using *t*-test; significances of relationships between $HCO_3^-$ to net C fixation and $CO_2$ concentrations were tested by means of linear regression.

## Results

### Growth characteristics

In both species, growth remained largely unaffected by changes in $pCO_2$ (Table 2), but *S. trochoidea* grew significantly faster than *A. tamarense* ($P < 0.001$) with average growth rates of $0.60 \pm 0.05$ $day^{-1}$ compared to $0.47 \pm 0.02$ $day^{-1}$, respectively. *Scrippsiella trochoidea* displayed a decrease in POC quota in response to elevated $pCO_2$ (Table 2), which was in line with a reduction in cell size (data not shown). In *A. tamarense*, POC quota remained unaltered over the applied $pCO_2$ range (Table 2), and were about twofold higher compared to *S. trochoidea*. Chl *a* quota in *S. trochoidea* showed maximum values in the 380 and 800 µatm $CO_2$ treatments, whereas in *A. tamarense*, they remained largely constant over the $pCO_2$ range (Table 2). Average Chl *a* quota were about sixfold higher in *A. tamarense* as compared to *S. trochoidea*. As a consequence of the

**Table 2.** Growth characteristics of *Scrippsiella trochoidea* and *Alexandrium tamarense* in the different $CO_2$ treatments. A significant difference between treatments is denoted by different letters. Values represent the mean ± SD of triplicate incubations (n = 3).

| $pCO_2$ (µatm) | Growth rate ($day^{-1}$) | POC production (ng $cell^{-1}$ $day^{-1}$) | POC production (pg (pg Chl *a*)$^{-1}$ day) | Chl *a* (pg $cell^{-1}$) | POC (ng $cell^{-1}$) | POC:PON (molar) | POC:Chl *a* (mass) |
|---|---|---|---|---|---|---|---|
| *S. trochoidea* | | | | | | | |
| 180 | $0.61 \pm 0.03$ | $1.21 \pm 0.04^{a}$ | $283 \pm 38^{a}$ | $4.3 \pm 0.71^{a}$ | $1.99 \pm 0.04^{a}$ | $7.6 \pm 0.2^{ac}$ | $469 \pm 81^{a}$ |
| 380 | $0.61 \pm 0.05$ | $1.08 \pm 0.08^{ab}$ | $143 \pm 13^{b}$ | $7.6 \pm 1.19^{ab}$ | $1.76 \pm 0.02^{ab}$ | $8.1 \pm 0.3^{ab}$ | $236 \pm 42^{b}$ |
| 800 | $0.61 \pm 0.04$ | $1.10 \pm 1.14^{a}$ | $127 \pm 20^{b}$ | $8.7 \pm 0.52^{b}$ | $1.79 \pm 0.22^{ab}$ | $8.4 \pm 0.3^{b}$ | $206 \pm 28^{b}$ |
| 1200 | $0.58 \pm 0.02$ | $0.87 \pm 0.02^{b}$ | $188 \pm 52^{b}$ | $4.9 \pm 1.25^{a}$ | $1.50 \pm 0.09^{b}$ | $7.4 \pm 0.1^{c}$ | $321 \pm 77^{ab}$ |
| *A. tamarense* | | | | | | | |
| 180 | $0.46 \pm 0.02^{ab}$ | $1.47 \pm 0.08$ | $40.5 \pm 3.9$ | $36.3 \pm 1.52$ | $3.17 \pm 0.25$ | $5.8 \pm 0.1$ | $88 \pm 11$ |
| 380 | $0.46 \pm 0.02^{ab}$ | $1.68 \pm 0.12$ | $42.0 \pm 4.6$ | $40.1 \pm 2.75$ | $3.62 \pm 0.31$ | $5.8 \pm 0.3$ | $91 \pm 9$ |
| 800 | $0.48 \pm 0.01^{a}$ | $1.67 \pm 0.06$ | $42.4 \pm 3.1$ | $39.5 \pm 3.34$ | $3.46 \pm 0.15$ | $5.7 \pm 0.1$ | $88 \pm 6$ |
| 1200 | $0.45 \pm 0.01^{b}$ | $1.55 \pm 0.06$ | $43.2 \pm 7.7$ | $36.4 \pm 5.82$ | $3.46 \pm 0.17$ | $5.6 \pm 0.1$ | $97 \pm 15$ |

differences in growth and POC quota, POC production rates of *S. trochoidea* decreased from 180 to 1200 µatm $pCO_2$ ($P = 0.005$; Table 2). *Alexandrium tamarense* displayed no changes in POC production rates toward elevated $pCO_2$ (Table 2), which were on average twice as high as in *S. trochoidea*.

The POC:PON ratio of *S. trochoidea* ranged between 7.4 and 8.4 with highest values in the 380 and 800 µatm $CO_2$ treatments, whereas the POC:Chl *a* ratio were highest in the 180 and 1200 µatm $CO_2$ treatments with $469 \pm 81$ and $321 \pm 77$, respectively (Table 2). In *A. tamarense*, POC:PON and POC:Chl *a* ratios remained unaffected by elevated $pCO_2$ with average values of $5.7 \pm 0.2$ and $90 \pm 10$, respectively. Calcification in *S. trochoidea* was very low with PIC:POC ratios <0.1 in all $CO_2$ treatments (data not shown), suggesting that calcite cyst formation in exponentially growing cells remains low (Wang et al. 2007).

## Oxygen and carbon fluxes

In *S. trochoidea*, net photosynthesis ($V_{max}$) and dark respiration remained largely unaltered between the different $CO_2$ treatments (Fig. 1). With mean values of $323 \pm 68$ and $173 \pm 21$ µmol $O_2$ (mg Chl *a*)$^{-1}$ $h^{-1}$, respectively, net photosynthetic rates were about twofold higher than dark respiration rates. In *A. tamarense*, net photosynthetic rates decreased from $142 \pm 4$ to $86 \pm 11$ µmol $O_2$ (mg Chl *a*)$^{-1}$ $h^{-1}$ ($P = 0.034$), while dark respiration rates increased from $88 \pm 5$ to $116 \pm 9$ µmol $O_2$ (mg Chl *a*)$^{-1}$ $h^{-1}$ from the lowest to the highest $CO_2$ treatment ($P = 0.009$).

*Scrippsiella trochoidea* preferentially took up $HCO_3^-$ with high affinities [i.e. low $K_{1/2}$ ($HCO_3^-$); Table 3]. In contrast, *A. tamarense* exhibited a high $CO_2$ uptake with high affinities [i.e. low $K_{1/2}$ ($CO_2$); Table 3]. Hence, the relative contribution of $CO_2$ and $HCO_3^-$ to net C fixation differed between the investigated species and

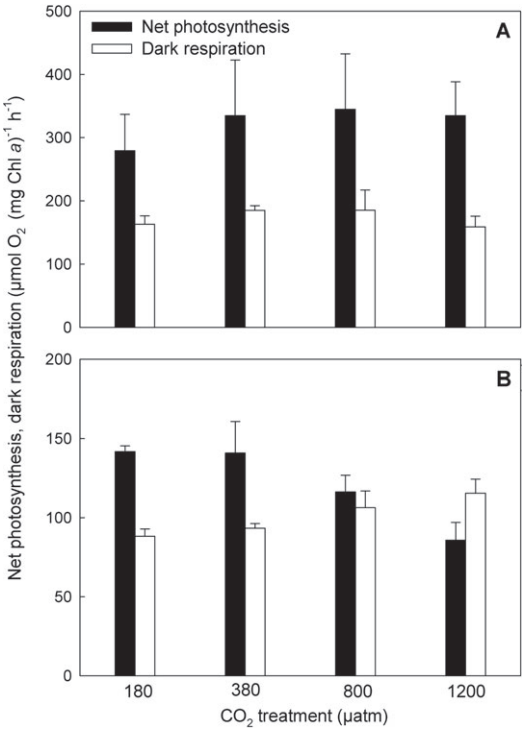


**Fig. 1.** Chl *a*-specific rates of net photosynthesis and dark respiration of *Scrippsiella trochoidea* (A) and *Alexandrium tamarense* (B) acclimated to different $CO_2$ concentrations. Bars represent mean ± SD (n = 3).

furthermore changed under elevated $pCO_2$. *Scrippsiella trochoidea* showed a decrease in the $HCO_3^-$ to net C fixation ratio from $0.99 \pm 0.17$ at the lowest to $0.70 \pm 0.14$ at the highest $CO_2$ treatment ($f = 1.065 - 0.0009x$; $R^2 = 0.48$; $P = 0.0128$; Fig. 2). *Alexandrium tamarense* used both $HCO_3^-$ and $CO_2$ as carbon source, with an increase in the $HCO_3^-$ to net C fixation ratio from $0.36 \pm 0.06$ at the lowest to $0.64 \pm 0.27$ at the highest $CO_2$ treatment ($f = 0.29 + 0.0003x$; $R^2 = 0.40$; $P = 0.0280$; Fig. 2).

**Table 3.** Net C fixation, net $CO_2$ uptake, $HCO_3^-$ uptake, eCA activity and leakage of *Scrippsiella trochoidea* and *Alexandrium tamarense* in the different $CO_2$ treatments. Values for $V_{max}$ and $K_{1/2}$ are given in μmol $mg^{-1}$ Chl *a* $h^{-1}$ and μmol $l^{-1}$, respectively. A dash indicates that values could not be determined. If not stated otherwise, values represent the mean ± SD of triplicate incubations (n = 3). A significant difference between treatments is denoted by different letters.

| | Net C fixation | | | Net $CO_2$ uptake | | $HCO_3^-$ uptake | | eCA activity | Leakage |
|---|---|---|---|---|---|---|---|---|---|
| $pCO_2$ (μatm) | $V_{max}$ | $K_{1/2}$ ($CO_2$) | $K_{1/2}$ (DIC) | $V_{max}$ | $K_{1/2}$ ($CO_2$) | $V_{max}$ | $K_{1/2}$ ($HCO_3^-$) | U (μg Chl *a*)$^{-1}$ | $CO_2$ efflux: total $C_i$ uptake |
| *S. trochoidea* | | | | | | | | | |
| 180 | 199 ± 41 | 3.8 ± 0.5 | 94 ± 50 | −13 ± 37[a] | – | 194 ± 5 | 7.2 ± 12.4 | 1573 ± 108 | 0.56 ± 0.06 |
| 380 | 239 ± 62 | 4.7 ± 1.1 | 160 ± 40 | −7 ± 29[a] | – | 225 ± 40 | 17 ± 1.4 | 1416 ± 22 | 0.53 ± 0.06 |
| 800 | 246 ± 62 | 5.5 ± 0.9 | 263 ± 51 | −3 ± 40[ab] | – | 202 ± 32 | 3.7 ± 5.5 | 1232 ± 144 | 0.54 ± 0.01 |
| 1200 | 239 ± 38 | 5.1 ± 0.4 | 269 ± 78 | 89 ± 22[b] | 20.1 ± 0.6 | 165 ± 26 | 10.3 ± 17 | 1301 ± 99 | 0.48 ± 0.04 |
| *A. tamarense* | | | | | | | | | |
| 180 | 101 ± 2[a] | 2.4 ± 0.1 | 267 ± 43 | 66 ± 9[a] | 3.3 ± 0.2 | 36 ± 5 | 105 ± 45 | 19 ± 48 (n = 2) | 0.44 ± 0.01[a] |
| 380 | 101 ± 14[ab] | 2.8 ± 0.2 | 309 ± 67 | 60 ± 16[ab] | 3.3 ± 0.3 | 38 ± 7 | 220 ± 96 | 86 ± 2 (n = 2) | 0.46 ± 0.02[a] |
| 800 | 83 ± 7[ab] | 2.0 ± 0.9 | 206 ± 65 | 42 ± 8[ab] | 3.1 ± 1.0 | 42 ± 10 | 158 ± 143 | 156 ± 12 (n = 2) | 0.53 ± 0.02[b] |
| 1200 | 61 ± 8[c] | 2.5 ± 0.2 | 173 ± 15 | 23 ± 26[b] | 4.5 ± 0.8 | 38 ± 13 | 148 ± 28 | 124 (n = 1) | 0.63 ± 0.05[c] |

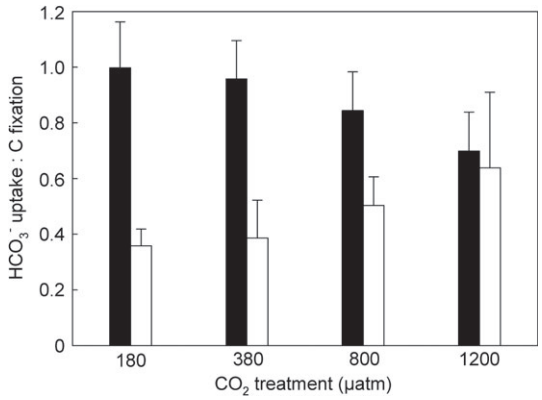


**Fig. 2.** Contribution of $HCO_3^-$ uptake relative to net C fixation of *Scrippsiella trochoidea* (black bars) and *Alexandrium tamarense* (white bars) acclimated to different $CO_2$ concentrations. Ratios were calculated using the Michaelis–Menten kinetics (Table 3) and the corresponding carbonate chemistry of the respective $CO_2$ treatments (Table 1). Bars represent mean ± SD (n = 3).

### Carbonic anhydrase activity

*Scrippsiella trochoidea* displayed exceptionally high eCA activities with up to 1600 U (μg Chl *a*)$^{-1}$ irrespective of the $CO_2$ treatments (Table 3). In contrast, *A. tamarense* contained relatively low eCA activities with mean values of 95 U (μg Chl *a*)$^{-1}$.

## Discussion

In this study, two bloom-forming dinoflagellate species with different traits, the calcifying *S. trochoidea* and the toxic *A. tamarense*, were exposed to a range of $pCO_2$ to investigate the effects of OA. While growth rates remained largely unaltered, elemental composition and production rates were responsive to OA. Both species also strongly regulated their underlying physiology with surprisingly different strategies to deal with changes in $CO_2$ supply.

### Growth and biomass production

Both species showed relatively small effects in terms of growth rates, yet we observed $CO_2$-dependent differences in POC production rates between species. In *S. trochoidea*, POC production rates decreased by almost 30%, which is reflected by a reduced cell size (data not shown) as well as lowered POC quota under elevated $pCO_2$ (Table 2). On the contrary, in *A. tamarense*, POC production rates and POC quotas remained largely unaltered (Table 2), the latter being similar to Leong et al. (2010). Average Chl *a* quota in *S. trochoidea* was largely comparable with earlier findings (Haardt and Maske 1987), whereas for *A. tamarense*, the average Chl *a* quota was about twice as high as earlier reported values (Carreto et al. 2001, Hu et al. 2006). Note that in none of the mentioned studies carbonate chemistry was controlled.

Regarding elemental composition, *S. trochoidea* showed highest POC:PON ratios under intermediate $CO_2$ concentrations (Table 2), with average ratios being lower than previously observed (approximately 9.3 in Burkhardt et al. 1999). These changes in POC:PON ratios are the result of disproportionally decreasing POC and PON quota under elevated $pCO_2$. In *A. tamarense*, POC:PON ratios were unaltered by the applied $CO_2$ treatments, and values were comparable with results of Leong et al. (2010). The significantly lower POC:PON ratio of *A. tamarense*, compared to *S. trochoidea*, may partly be attributed to the fact that it produces nitrogen-rich paralytic shellfish poisoning toxins (PST; Bates et al. 1978). However, the overall contribution of PST to

total cellular nitrogen for this strain of *A. tamarense* accounts for less than 4% (Van de Waal et al. 2014), and thus cannot alone explain the observed differences in POC:PON between both species.

In contrast to our expectations, processes like growth and elemental ratios were not strongly affected by OA. With respect to POC production, however, species differed in their responses, which could be attributed to $CO_2$-dependent changes in photosynthesis, in particular in their mode of $C_i$ acquisition. We therefore performed MIMS measurements targeting those underlying processes.

### Photosynthesis and respiration

In *S. trochoidea*, rates of $O_2$ evolution (i.e. net photosynthesis) were more than twofold higher than in *A. tamarense*, which is in line with higher growth rates as well as higher POC:Chl *a* ratios (Table 2). Both species exhibited high dark respiration rates compared to net photosynthetic rates (Fig. 1). Provided that measured respiration during darkness is representative also for the light phase, respiration was approximately 50% of net photosynthesis in *S. trochoidea*, whereas in *A. tamarense* both rates were equally high. Comparable high dark respiration rates have since long also been shown for other dinoflagellate species, including zooxanthellae (e.g. Burris 1977). In *A. tamarense*, net photosynthesis and respiration furthermore showed opposing trends in response to elevated $pCO_2$. The decrease in net photosynthesis in *A. tamarense* may be largely caused by the increased dark respiration under elevated $pCO_2$. Other processes affecting $O_2$ uptake in the light, such as Mehler Reaction and photorespiration, can however not be excluded here and may potentially alter the trends. Brading et al. (2013), for example, observed significant light-dependent $O_2$ uptake in four *Symbiodinium* strains, which remained unaltered under OA. Other studies showed that OA effects can be modulated under different light levels and may enhance mitochondrial respiration, photorespiration and ultimately reduce growth and biomass production under high light (Gao et al. 2012, Rokitta and Rost 2012, Li and Campbell 2013). Interestingly, the increase in respiration with $pCO_2$ observed in *A. tamarense* was found to have no effect on growth or POC production rates. Overall, it can be concluded that the sum of net photosynthesis and respiration, i.e. gross photosynthesis, remained largely unaffected in both tested species.

Previous studies on *Protoceratium reticulatum* and four strains of *Symbiodinium* showed basically no $CO_2$ effect on photosynthesis and respiration (Ratti and Giordano 2007, Brading et al. 2011), with the exception of one *Symbiodinium* strain that showed higher rates of net photosynthesis under OA (Brading et al. 2011). Interestingly, in *Protoceratium reticulatum* and another *Symbiodinium* strain, growth nonetheless increased with elevated $pCO_2$ (Ratti and Giordano 2007, Brading et al. 2011). These findings, together with our current results, demonstrate that responses in growth and biomass production toward OA cannot always be explained by changes in $O_2$ fluxes, but instead may be attributed to the mode of $C_i$ acquisition. High sensitivities in growth and biomass production toward OA, for instance, have often been associated with a strong dependency on $CO_2$ as a $C_i$ source for photosynthesis (Colman et al. 2002, Fu et al. 2008), whereas when $HCO_3^-$ is the dominant $C_i$ source, much less sensitivity toward changes in $CO_2$ is expected (Burkhardt et al. 1999, Rost et al. 2008). Therefore, we assessed various key components of the CCM and their potential $CO_2$-dependent regulation to understand the responses of *S. trochoidea* and *A. tamarense* toward OA.

### Carbon source and carbonic anhydrase

Among the various studies on carbon acquisition in dinoflagellates, either $CO_2$ (Colman et al. 2002, Dason et al. 2004, Fu et al. 2008, Lapointe et al. 2008, Brading et al. 2013) or $HCO_3^-$ (Rost et al. 2006, Ratti and Giordano 2007, Fu et al. 2008) was estimated to be the dominant $C_i$ source. Here we show that *S. trochoidea* and *A. tamarense* used $CO_2$ as well as $HCO_3^-$ for photosynthesis, though their contribution to net C fixation and response to elevated $pCO_2$ were very different. As one would expect, *S. trochoidea* displayed an increase in relative $CO_2$ uptake, or in other words, a decrease in relative $HCO_3^-$ uptake to net fixation under elevated $pCO_2$ (Fig. 2). Such a trend has also been observed in other functional groups, e.g. diatoms (Burkhardt et al. 2001, Trimborn et al. 2009, 2013), coccolithophores (Rost et al. 2003) or cyanobacteria (Kranz et al. 2009). The response in *A. tamarense*, however, was surprising as it showed the reverse trend, i.e. an increase of $HCO_3^-$ uptake in response to elevated $pCO_2$ (Fig. 2). This could be associated with the generally high and increasing rates of respiration and $CO_2$ efflux observed in this species (Fig. 1, Table 3). $HCO_3^-$ uptake may therefore be simply upregulated to compensate for the increasing $CO_2$ efflux. Even though respiration can partly cause the high loss of $C_i$ from the cell, it could be speculated that the increase in respiration may also provide the required ATP to fuel the higher $HCO_3^-$ uptake. Why mitochondrial activity, in the first place, is stimulated under OA scenarios remains elusive, but it could be associated to altered proton gradients across the mitochondrial membrane or to pH-dependent

changes in the functioning of respiratory enzymes (Amthor 1991).

According to the common notion, eCA functions to replenish the $CO_2$ pool in the $CO_2$ depleted boundary layer of a cell, thereby fuelling the $CO_2$ uptake systems (Badger and Price 1989, Sültemeyer 1998, Elzenga et al. 2000). Such mechanism would obviously be most effective when a cell predominantly uses $CO_2$ as its $C_i$ source. For dinoflagellates, a major role of eCA activity in CCM functioning was only indicated for the $CO_2$ user *Lingulodinium polyedrum* and *Symbiodinium* A20 (Lapointe et al. 2008, Brading et al. 2013). Activities of eCA in most other tested dinoflagellates, including *A. tamarense* in this study, were close to detection limits and therefore likely play only a minor role, if any, in $C_i$ acquisition (Table 3; Colman et al. 2002, Rost et al. 2006, Ratti and Giordano 2007). In *S. trochoidea*, however, we observed exceptionally high eCA activities of up to 1600 U (μg Chl *a*)$^{-1}$ over the entire $pCO_2$ range (Table 3). Why would a predominant $HCO_3^-$ user have such high eCA activities? Comparable high eCA activities in concert with high $HCO_3^-$ contribution have been observed previously (Martin and Tortell 2008, Trimborn et al. 2008, 2013), and our observation that eCA and $HCO_3^-$ uptake are both upregulated at high pH casts further doubts on an universal role of eCA.

Trimborn et al. (2008) proposed that in $HCO_3^-$ users, eCA may convert effluxing $CO_2$ to $HCO_3^-$, which is subsequently taken up again by the cell. Such a '$CO_2$ recycling mechanism' would be particularly advantageous for species with high respiration rates, which was indeed the case for *S. trochoidea* (Fig. 1). For *Thalassiosira* spp., however, the effectiveness of such a mechanism was recently questioned as it would increase the $C_i$ uptake rate by less than 1% only (Hopkinson et al. 2013). This situation may, however, strongly differ between species as model estimates depend on the net $CO_2$ uptake, which is large for *Thalassiosira* spp. (Hopkinson et al. 2013) but not for *S. trochoidea* (Table 3). In fact, net $CO_2$ uptake in *S. trochoidea* was close to zero or even negative and there was a high leakage, i.e. about 50% of the $C_i$ taken up by the cell was leaking out as $CO_2$ (Table 3), which is not accounted for in the model calculations (Hopkinson et al. 2013). Particular high leakage has also been measured in other dinoflagellates (Rost et al. 2006). It should be noted, however, that $C_i$ fluxes are typically determined using disequilibrium approaches and thus require the inhibition of potential eCA activity (Badger et al. 1994). If eCA activity would indeed be involved in minimizing the $CO_2$ efflux, this approach may overestimate leakage for *S. trochoidea*, while estimates in *A. tamarense*, which lacks eCA activity, would not be biased by the approach. In any case, although eCA presumably contributes to the CCM, its role and correlation with high $HCO_3^-$ uptake remains puzzling and requires further investigations.

## CCMs and trade-offs within

With respect to net C fixation, both *S. trochoidea* and *A. tamarense* displayed half-saturation concentrations ($K_{1/2}$) of <6 μmol $CO_2$ l$^{-1}$ at all applied $CO_2$ levels (Table 3). These results were consistent with previously published $K_{1/2}$ values of other dinoflagellates (Rost et al. 2006, Ratti and Giordano 2007) and fall in the same range as those measured for temperate diatoms (Burkhardt et al. 2001, Trimborn et al. 2008, 2009, Yang and Gao 2012), which are known to feature very effective CCMs (Reinfelder 2011 for review). Interestingly, the $K_m$ value of the type II RubisCO employed in dinoflagellates (80–250 μmol $CO_2$ l$^{-1}$) is much higher than the $K_m$ of type I in diatoms (31–41 μmol $CO_2$ l$^{-1}$; Badger et al. 1998). In other words, the CCM in these dinoflagellates increased not only their $CO_2$ affinities by more than one order of magnitude relative to their RubisCO kinetics, but also demonstrates that the activity of the CCM in dinoflagellates must be up to sixfold higher than that of diatoms. Additionally, dinoflagellate cells are typically larger than diatoms, which automatically reduces the surface to volume ratio and hence the specific reaction diffusion-supply rate of $CO_2$ to the cell surface (Reinfelder 2011). The correspondingly higher energy expenditure for running their CCM could thus, to a large degree, explain why dinoflagellates grow generally much slower than diatoms and thrive under different environmental conditions (Smayda 1997).

Next to the $K_{1/2}$ value, also the maximum rate ($V_{max}$) plays an important role in determining the competitive success of a species (Healey 1980). Interestingly, our data indicate a trade-off between $V_{max}$ and $K_{1/2}$ values between both species. *Scrippsiella trochoidea* displayed relatively high $V_{max}$ and high $K_{1/2}$ values, while *A. tamarense* showed the inverse pattern, i.e. relatively low $V_{max}$ and low $K_{1/2}$ (Fig. 3). The observed trade-off within the kinetic properties of $C_i$ acquisition is also present between the different $CO_2$ treatments, especially for *S. trochoidea* showing a relative decrease in affinities with increasing maximum rates. This correlation may reflect fundamental characteristics of nutrient uptake in microalgae (Raven 1980, Aksnes and Egges 1991, Lichtman et al. 2007). Given the limited area of the cell's surface available for nutrient uptake, the number of transporters with small active area per transporter (leading to higher $V_{max}$ and higher $K_{1/2}$) 'compete' with the number of uptake sites with relatively large active areas (leading to lower $V_{max}$ and lower $K_{1/2}$).

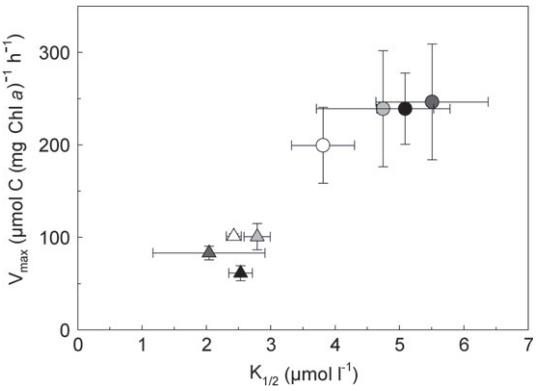


**Fig. 3.** $V_{max}$ vs $K_{1/2}$ of photosynthetic carbon fixation of *Scrippsiella trochoidea* (circles), *Alexandrium tamarense* (triangles) acclimated to different $CO_2$ concentrations. Color of symbols indicates $CO_2$ treatments from low (white) to high (black). Symbols represent mean ± SD (n = 3).

The fact that cells do not come up with transporters being characterized by high $V_{max}$ as well as low $K_{1/2}$ is probably dictated by biochemical constraints, i.e. transporters can be faster only at the expense of lower affinities or vice versa (Fersht 1974). Our findings on the trade-off between $V_{max}$ and $K_{1/2}$ in $C_i$ acquisition are in line with previously observed characteristics on N acquisition in the major eukaryotic phytoplankton groups (Lichtman et al. 2007) as well as different strains of two $N_2$ fixing cyanobacteria species (Hutchins et al. 2013). However, whether this trade-off in $C_i$ acquisition is a general feature holding true also for other species and strains (Brading et al. 2013 show $V_{max}$ and $K_{1/2}$ values of two *Symbiodinium* strains being similar to *S. trochoidea*) and even taxa needs to be further investigated.

## Ecological implications

To compensate potential limitations in the carboxylation reaction of RubisCO, *S. trochoidea* and *A. tamarense* operate effective CCMs, allowing both species to grow unaltered over the applied range of $pCO_2$. More specifically, both species substantially increased their overall affinities for photosynthesis, relative to what would be predicted by RubisCO, and were also able to use $HCO_3^-$ as $C_i$ source. However, the high levels of $C_i$ accumulation required for the low affine RubisCO, the predominant $HCO_3^-$ uptake, as well as the high $CO_2$ leakage cause $C_i$ acquisition to be very costly, which may have profound ecological consequences. For instance, it could partly explain why dinoflagellates display generally lower growth rates compared to other major groups of marine phytoplankton, which employ a more affine type I RubisCO (Smayda 1997). Reasons why dinoflagellates yet thrive well in many environments can partly be attributed to their mixotrophic behavior (Jeong et al. 2005), the potential of some species to produce allelopathic compounds (Cembella 2003) and the ability to migrate within the water column to circumvent nutrient and light limitation (MacIntyre et al. 1997). Active swimming may as well lower diffusion limitation (Pahlow et al. 1997), in particular for nutrients like nitrate or trace elements, but it could also enhance the $CO_2$ supply to the cell surface and thereby possibly reduce the costs of CCMs in dinoflagellates.

The observed trade-off between maximum uptake rates and affinities for $CO_2$ may also play a role in optimizing the competitive success of both species at different $CO_2$ levels. More specifically, having a higher $V_{max}$ and higher growth rate, *S. trochoidea* exhibits the 'velocity' strategy (Sommer 1984), which will be favored under high and dynamic $C_i$ availabilities. With a lower $K_{1/2}$, on the other hand, *A. tamarense* exhibits an 'affinity' strategy (Sommer 1984) that will have a competitive advantage under low $C_i$ concentrations (Fig. 3, Table 3). During phytoplankton blooms, carbonate chemistry may substantially change and drift toward high pH and low $CO_2$ concentrations (Hansen 2002). As a consequence, species with a low $K_{1/2}$ for $CO_2$, such as *A. tamarense*, may be favored. At the same time, however, carbonate chemistry may also exhibit strong daily fluctuations as results of day-time photosynthesis and night-time respiration. Under these conditions, species with a high $V_{max}$ and growth rate, like *S. trochoidea*, are likely to be favored. On top of that, the high preference of *S. trochoidea* for $HCO_3^-$ may further support its growth during blooms. It thus seems that *A. tamarense* and *S. trochoidea* exhibit different strategies allowing them to cope with dense bloom conditions. Such differences in competitive strategies, induced by physiological characteristics, may furthermore allow coexistence of multiple species.

Species being able to regulate their CCM in response toward high $pCO_2$ and low pH conditions will have advantages in a future ocean. Even though both tested species were regulating their CCM, *S. trochoidea* showed strongest changes in response to OA. Modes of CCMs and thus $CO_2$ sensitivities in growth and biomass production may, however, change strongly under resource limitation, i.e. nutrient depleted or low light conditions, and therefore alter the outcome of competition under OA. For bloom-forming species like *S. trochoidea* and *A. tamarense*, which tend to flourish late in the succession, investigations on the interactive effects of nutrient limitation and OA as well as dynamic changes therein are crucial to improve our understanding of the response of this important group of phytoplankton in a future, high $CO_2$ world.

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