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The combined effect of pH and dissolved inorganic carbon concentrations on the physiology of plastidic ciliate *Mesodinium rubrum* and its cryptophyte prey

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Ocean acidification is caused by rising atmospheric partial pressure of CO₂ (pCO₂) and involves a lowering of pH combined with increased concentrations of CO₂ and dissolved in ocean waters. Many studies investigated the consequences of these combined changes on marine phytoplankton, yet only few attempted to separate the effects of decreased pH and increased pCO₂. Moreover, studies typically target photosynthetic protists that depend on the ingestion of plastids from their prey. Therefore, we studied the separate and interactive effects of pH and DIC levels on the plastidic ciliate *Mesodinium rubrum*, which is known to form red tides in coastal waters worldwide. Also, we tested the effects on their prey, which typically are cryptophytes belonging to the *Teleaulax/Plagiolenmis/Geminigera* species complex. These cryptophytes not only serve as food for the ciliate, but also as a supplier of chloroplasts and prey nuclei. We exposed *M. rubrum* and the two cryptophyte species, *T. acuta*, *T. amphioxia* to different pH (6.8 - 8) and DIC levels (~6.5 - 26 mg C L⁻¹) and assessed their growth and photosynthetic rates, and cellular chlorophyll a and elemental contents. Our findings did not show consistent significant effects across the ranges in pH and/or DIC, except for *M. rubrum*, for which growth was negatively affected only by the lowest pH of 6.8 combined with lower DIC concentrations. It thus seems that *M. rubrum* is largely resilient to changes in pH and DIC, and its blooms may not be strongly impacted by the changes in ocean carbonate chemistry projected for the end of the 21st century.

1. Introduction

Ocean acidification (OA) refers to a reduction in pH of ocean waters as a result of rising atmospheric CO₂ levels in the environment (Gattuso and Hansson, 2011). Atmospheric CO₂ levels have been estimated to increase from ~280 ppm in the mid-18th century to ~1000 ppm at the end of 21st century (IPCC 2021). This increase in atmospheric CO₂ partial pressure (pCO₂) will also lead to an increase in the uptake of CO₂ by the oceans. Currently, the oceans absorb ~25% of the CO₂ emitted by human activities and the burning of fossil fuels. This increased uptake of atmospheric CO₂ into the ocean will alter the concentration and specification of dissolved inorganic carbon (DIC) and lead to a decrease in seawater pH. It has been hypothesized that the average ocean surface water pH will decrease from ~8.2 to < 7.7 by the end of 21st century (Hoegh-Guldberg et al., 2014; IPCC 2021).

The pH of seawater depends on the carbonate system and, specifically, the concentrations of the different forms of inorganic carbon (Millero, 2000). With increasing atmospheric pCO₂, more CO₂ can react with water and form carbonic acid (H₂CO₃). This dissociates into bicarbonate (HCO₃⁻) and releases a proton (H⁺). Depending on the solubility of CO₂, alkalinity and the concentration of DIC, the HCO₃⁻ may further dissociate into carbonate (CO₃²⁻), releasing another H⁺. Consequently, with higher levels of CO₂, more protons are formed, and hence the pH of the water decreases. Vice versa, when CO₂ is taken up from the water by primary production, less protons are formed, and hence the pH increases. This can lead to strong seasonal dynamics in the carbon
chemistry, particularly in productive coastal waters (Hansen, 2002).

Large seasonal and decadal variations in pH in temperate coastal waters are mainly caused by two phenomena: 1) high winter concentrations of nutrients that can lead to a phytoplankton spring bloom with enhanced primary production, resulting in elevated pH, and 2) the low DIC present due to freshwater runoff in coastal waters (Thomas and Schneider 1999; Key et al., 2004), resulting in lower pH buffer capacity. Studies on seasonal variation in eutrophic coastal fjord and embayments have reported fluctuations in surface pH range of 6.9 - 9.5, with the lowest and highest pH values being found during winter and spring/summer, respectively (Hansen, 2002; Carstensen et al., 2018). Decadal changes have also been observed in many different coastal waters worldwide that far exceed the predicted global predictions of ocean acidification in surface waters (Carlsten and Duarte, 2018; Carlsten et al., 2018).

Many OA studies of marine planktonic primary producers have only included pH/CO2 levels at present conditions of ~pH 8.2 and pCO2 in the range of 360 - 400 ppm, and a so-called future treatment, typically with pH values down to 7.8 and pCO2 in the range of 750–1000 ppm. Yet, this ignores the non-linearity of many of these responses, and findings cannot be extrapolated to natural coastal waters that exhibit large decadal and seasonal variations. Thus, there is a need to conduct studies at a wider range of pH and pCO2 levels (Van de Waal et al., 2021).

Changes in pCO2 and pH may affect planktonic primary producers in various ways. Higher CO2 concentrations due to lower pH may fuel photosynthesis and possibly support faster growth rates. However, often marine photosynthetic primary producers are not limited by pCO2, as many species exhibit effective carbon concentrating mechanisms that allow active uptake of CO2 as well as HCO3-, where especially the latter is already available in ample supply in seawater. At a pH 8 - 8.2, the greatest part of C-species is in the form of HCO3 (~90%), and only ~1% (10 to 20 μM) are in the form of CO2 (Millero, 2000). Yet, down-regulation of carbon concentrating mechanisms (CCMs) with elevated pCO2 may possibly lead to reallocation of resources from C acquisition to other processes (Van de Waal et al., 2019; Van de Waal and Litchman, 2020). Whether increased pCO2 will thus affect growth rate will largely depend on the ability of species to regulate their CCMs and increase their overall C acquisition efficiency. Besides changes in pCO2 decreases in pH may also directly affect phytoplankton.

Studies of OA on marine planktonic primary producers in the laboratory have so far only been done on primary producers that have chloroplasts of their own, whether or not they can be mixoplanktonic (combining photosynthesis and food uptake). However, many photosynthetic protists are dependent on the sequestration of chloroplasts from the prey; they are often referred to as kleptoplastidic protists, and can be found among ciliates, dinoflagellates, and Rhizaria (Stoecker et al., 2017). Ciliates belonging to the *Mesodinium rubrum/major* species complex are examples of such kleptoplastidic protists (Johnson et al., 2016; Drumm et al., 2021). They form blooms in coastal waters worldwide (Lindholm, 1985), and even occur in highly brackish waters, like the inner Baltic Sea at salinities of < 5 and DIC concentrations of ~14 mg C L−1 (Lindholm and Mörk, 1990). *Mesodinium* is considered a harmful algal bloom (HAB) species. Although it is not toxic it causes blooms, e.g. red-tides, which can lead to oxygen depletion. These ciliates depend on specific cryptophytes belonging to the *Teleaulax/Plagioelmis/Geminigera* complex for the supply of food and chloroplasts. Ingestion rates are typically low, and at high irradiances, > 96% of the carbon uptake derives from photosynthesis (Smith and Hansen, 2007). The reason for choosing *Teleaulax* and *Mesodinium* was based on the attempt to explore the response of the important food chain *Teleaulax-Mesodinium-Dinophys* in relation to OA.

We currently know very little about the responses of species from the *M. rubrum/major* species complex, and their prey towards OA. A few studies have indicated that *M. rubrum* is quite sensitive to elevated pH, and that growth rates are affected already when pH exceeds 8.5 (Hansen and Fenchel, 2006; Smith and Hansen, 2007). However, how these ciliates are affected by reduced pH values associated to OA is presently unknown. A couple of studies have investigated the effects of lowered pH on the growth of the cryptophyte *Teleaulax amphioxeia* (Berge et al., 2010; Gaillard et al., 2020). These studies show that *T. amphioxeia* seems to be largely unaffected in growth and chlorophyll a content at a pH range of 8.0 down to 6.4, and growth only decreased at lower pH values. The question remains how sensitive this species is to changes in the availability of DIC, and particularly when changes in pH and DIC concentrations occur separately or in combination.

The overall aim of this study was therefore to investigate how growth and photosynthetic rates, as well as elemental contents and ratios of the kleptoplastidic ciliate *M. rubrum* and its cryptophyte prey, *Teleaulax acuta* and *T. amphioxeia*, respond to a wide range of pH values and DIC concentrations to address both the separate and combined effects of acidification and changes in inorganic carbon availability. We chose to expose the organisms to pH in the range of 6.8 - 8.0 in the DIC range of ~6.5 - 26 mg C L−1, thereby covering, and slightly exceeding, the lower levels that these organisms may experience in their natural environment (Hansen, 2002; Carstensten et al., 2018; Carlsten and Duarte, 2018).

Our hypotheses were:

1. The rates of photosynthesis and growth of the cryptophytes are not expected to be affected by lowered pH because of their wide pH tolerance, but they will be affected by low DIC as this may induce C limitation (<13 mg C L−1)

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**Table 1**

P-values (before Holm adjustment) from hypothesis tests for DIC, pH and pCO2 effects on rates of growth rates, cellular Chl a content, rates of photosynthesis, cellular carbon content and C:N and C:P ratios of *T. acuta*, *T. amphioxeia* and *M. rubrum*. Very small P values are indicated as *P* < 0.0001. Holm adjustment for multiple testing was carried out for each type of test (each column with P-values), and effects are regarded significant if Holm adjusted P-values (not shown) are < 0.05 and are marked with “*”, “**” and “***” if adjusted *P* < 0.05, between 0.01 and 0.05, and < 0.01, respectively.

<table>
<thead>
<tr>
<th>Figure number</th>
<th>Species</th>
<th>Response</th>
<th>Interaction between pH and DIC</th>
<th>Non-linearity</th>
<th>Overall pH</th>
<th>Overall DIC</th>
<th>Overall pCO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 1a,b</td>
<td><em>T. acuta</em></td>
<td>Growth rate</td>
<td>0.28</td>
<td>0.47</td>
<td>0.00017**</td>
<td>0.36</td>
<td>0.027</td>
</tr>
<tr>
<td>Fig. 1c,d</td>
<td><em>T. amphioxeia</em></td>
<td>Growth rate</td>
<td>0.0026*</td>
<td>0.59</td>
<td>0.0021*</td>
<td>0.63</td>
<td>0.10</td>
</tr>
<tr>
<td>Fig. 1e</td>
<td><em>M. rubrum</em>, all</td>
<td>Growth rate</td>
<td>&lt; 0.001***</td>
<td>0.017</td>
<td>&lt; 0.0001***</td>
<td>0.00041**</td>
<td>0.87</td>
</tr>
<tr>
<td>Fig. 1f</td>
<td><em>M. rubrum</em>, w/o pH 6.8</td>
<td>Growth rate</td>
<td>0.10</td>
<td>&lt; 0.001***</td>
<td>0.67</td>
<td>0.00069**</td>
<td></td>
</tr>
<tr>
<td>Fig. 2a,b</td>
<td><em>T. amphioxeia</em></td>
<td>Chl a</td>
<td>0.036</td>
<td>0.434</td>
<td>&lt; 0.0001***</td>
<td>0.10</td>
<td>0.80</td>
</tr>
<tr>
<td>Fig. 2c,d</td>
<td><em>T. amphioxeia</em></td>
<td>Photosynthesis</td>
<td>0.0021*</td>
<td>0.204</td>
<td>&lt; 0.001***</td>
<td>0.30</td>
<td>0.12</td>
</tr>
<tr>
<td>Fig. 3a,b</td>
<td><em>T. amphioxeia</em></td>
<td>Carbon content</td>
<td>0.034</td>
<td>0.204</td>
<td>&lt; 0.001***</td>
<td>0.30</td>
<td>0.12</td>
</tr>
<tr>
<td>Fig. 3c,d</td>
<td><em>T. amphioxeia</em></td>
<td>Chl a</td>
<td>0.034</td>
<td>0.204</td>
<td>&lt; 0.001***</td>
<td>0.30</td>
<td>0.12</td>
</tr>
<tr>
<td>Fig. 4a</td>
<td><em>M. rubrum</em>, w/o pH 6.8</td>
<td>Chl a</td>
<td>&lt; 0.0001***</td>
<td>0.047</td>
<td>&lt; 0.0001***</td>
<td>0.30</td>
<td>0.12</td>
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<tr>
<td>Fig. 4b</td>
<td><em>M. rubrum</em>, w/o pH 6.8</td>
<td>Photosynthesis</td>
<td>0.38</td>
<td>0.18</td>
<td>0.0001***</td>
<td>0.77</td>
<td>0.96</td>
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<tr>
<td>Fig. 4c,d</td>
<td><em>M. rubrum</em>, w/o pH 6.8</td>
<td>Carbon content</td>
<td>0.012</td>
<td>0.029</td>
<td>&lt; 0.0074*</td>
<td>&lt; 0.001***</td>
<td>0.0035*</td>
</tr>
<tr>
<td>Fig. 5a,b</td>
<td><em>M. rubrum</em>, w/o pH 6.8</td>
<td>Chl a</td>
<td>&lt; 0.0001***</td>
<td>0.025</td>
<td>&lt; 0.0145*</td>
<td>0.11</td>
<td>0.039</td>
</tr>
<tr>
<td>Fig. 5c,d</td>
<td><em>M. rubrum</em>, w/o pH 6.8</td>
<td>Chl a</td>
<td>&lt; 0.0001***</td>
<td>0.036</td>
<td>&lt; 0.0035*</td>
<td>0.09</td>
<td>0.68</td>
</tr>
</tbody>
</table>
The rates of photosynthesis and growth of *M. rubrum* are reduced at very low pH values (<pH 7) as these will be stressful to the cells, and low DIC concentrations as this may induce C limitation (<13 mg C L\(^{-1}\)).

Both the ciliate and the cryptophytes will decrease their cellular C:N and C:P ratios when exposed to low DIC as this may limit C fixation, especially at the higher pH values in the range of 7.7–8.0 where CO\(_2\) concentrations will be lower.

### 2. Material and methods

#### 2.1. Experiment 1. The growth rate at different pH and DIC treatments

#### 2.1.1. Organisms and culture conditions

Three primary producers were included in this study, two cryptophytes, *T. acuta* (K-1486) and *T. amphioxeia* (K-1837), and one ciliate, *M. rubrum* (MBL-DK2009). *T. acuta* was established from Nivå Bay Denmark, 2009, and *T. amphioxeia* and *M. rubrum* were collected from Elsinore Harbor Denmark in 2009. Both *Teleaulax* spp. are deposited in The Norwegian Culture Collection of Algae, NORCCA. All species were
maintained in f/20 medium, based on filtered seawater collected outside Elsinore Harbor with a salinity of 31 and temperature of 15°C. The cultures were kept under a light intensity of 145–180 µmol photons m⁻² s⁻¹ in a light:dark cycle of 14:10 h.

2.1.2. The experimental treatments

The organisms were acclimated to the specific treatment conditions for seven days prior to the measurements in 250 mL batches. On day five of the acclimation period, the cultures were separated into triplicates in 500 mL borosilicate flasks. The cryptophytes were always kept in monocultures, while *M. rubrum* was kept in mixed cultures, where they were fed with acclimated prey (*T. amphioxeia*) during the acclimation period, with a feeding ratio 4:1, so no prey was left when entering the experimental period.

The different DIC concentrations were selected to test if there were any impacts of lowered DIC, and thereby potential inorganic carbon limitation, on the given measured ecophysiological parameters (growth rate, cellular Chl a contents and the photosynthetic rate). The DIC levels of interest were 6.5, 13 and 26 mg C L⁻¹. The standard f/20 media had a DIC level at approximately 26 mg C L⁻¹, representing the typical oceanic DIC concentration. The respective DIC values for the treatments were obtained by diluting the standard growth medium with medium lacking DIC. To achieve seawater without any DIC, we added acid (HCL) to freshly filtered seawater to reach pH 3, and subsequently bubbled the seawater with atmospheric air to get rid of the DIC pool in the water. Thereafter, we brought the pH back to the desired level by adding NaOH.

Enumeration of live cryptophytes was done using a FlowCytometer (Beckmann Coulter - CytoFLEX), with few exceptions, where the enumerations were done using a Coulter Counter (Beckman Coulter - Multisizer 3). The ciliate *M. rubrum* could not be enumerated by the flow cytometer. It was instead fixed in Acidic Lugol’s iodine solution and enumerated using a Sedgewick-Rafter chamber, for which at least 200 cells were counted each time. The growth rate measurements started after the acclimation period had ended, at the same time each day. During the growth experiments, the cells were enumerated daily or bi-daily to achieve 3 - 4 data points for each treatment.

The day-to-day growth rate (µ') was measured as increase in the cell concentration, and calculated as

\[ \mu' (d^{-1}) = \frac{lnN_1 - lnN_0}{t_1 - t_0} \]  \hspace{1cm} (1)

where \(N_0\) and \(N_1\) are the number of cells before dilution \(t_1\) and after previous dilution \(t_0\), respectively. To adjust for the daily dilution, cumulative cell abundance \(N_{cum}\), was estimated according to the formula

\[ N_{cum} \text{ (cells mL}^{-1}) = N^0 \cdot e^{\mu'(t_1 - t_0)} \]  \hspace{1cm} (2)

where \(N^0\) is the cell concentration at \(t_0\) before previous dilution. Overall growth rates (µ, D⁻¹) in the different treatments were calculated as the slope of the linear regression of the ln-transformed \(N_{cum}\) data as a function of time including three to four sampling time points for each
replicate bottle, respectively.

2.1.3. Measurements and adjustments of pH and DIC

The pH in the experimental treatments was measured by a pH meter (WTW 3210), equipped with a Sentix 31 pH electrode, and was adjusted to keep the individual treatments at \( \pm 0.05 \) from set-point in the large majority of cases (see exceptions in Supplementary Table 1). The pH meter went through a 2-point calibration once a week using pH 7 and pH 10 NIST buffers (NBS-scale). The pH, DIC and cell concentrations were adjusted by daily dilutions with pH and DIC adjusted media, to keep stable values.

The pH of f/20 medium was manipulated by addition of 0.1 or 1.0 M NaOH or HCl. The acid/base addition in the DIC 13 and DIC 6.5 media was added in a subsample of the media and then mixed with the rest of the media. This was done so the acid and base addition was held at the lowest amount possible, to minimize the waste of water and minimize the oscillations of pH in the cultures.

The DIC levels in each culture were analyzed using a Total Organic Carbon analyzer (TOC-L CSN, Shimadzu). To this end, 25 mL was withdrawn from each sample and a 5-point calibration was made each week, ranging from 2.5 to 30 mg C L\(^{-1}\). The measured DIC was then used for TCO\(_2\), thereby allowing the calculation of pCO\(_2\) by the CO2sys_v2.3 program (Pierrot et al., 2006).

2.2. Experiment 2. T. amphioxeia and M. rubrum - Photosynthetic rates, cellular Chl a content and C:N:P stoichiometry at different pH and DIC treatments

The culture conditions applied the same experimental approach for experiment 2, as for experiment 1, also the setup of DIC measurements and sampling methods was identical to the first experiment. The number of samples and the volume differed. The samples were taken on day 1 after the acclimation period, hence all samples for experiment 2 were taken on the same day.

Chlorophyll \( \alpha \) analysis was carried out on samples collected from each treatment. For this, 10 mL subsamples were withdrawn and added to Falcon tubes (15 ml) and subsequently filtered onto GF/F glass fiber filters (25 mm). A compressor (pressure < 5 Hg) was used in the case of the cryptophytes, while a hand pump was used for \( M. \) rubrum. The filters were then extracted in 5 ml 96% ethanol and stored in the refrigerator for 24 h. The Chl \( \alpha \) was quantified using a Trilogy Fluorometer (Turner Designs), equipped with a Chl-NA (Chlorophyll Non-Acid) insert.

For measurements of the photosynthetic rate, two sets of 20 mL subsamples were withdrawn from each treatment and added to 20 mL glass vials. To each vial, 20 \( \mu \)L of NaH\(^{14}\)CO\(_3\) was added, except at pH 6.8 where it was added in a lower concentration. The reason for this was the lowered buffer capacity and the addition of C\(^{14}\)-labelled HCO\(_3\) causes greater oscillations in pH and hence the dissociation of protons. One set of vials were then incubated for 3 h in the light at the same irradiance for
experiment 1 (light vials), while a separate set of vials were incubated in the dark (dark vials), to correct for dark uptake of C\textsuperscript{14}. After the incubation 5 mL of each incubated sample was added to new vials, and 5 mL of 10% glacial acetic acid in methanol was added. The vials were transferred to heating-plates (65 °C) overnight until they dried out. 3 mL of distilled water and 10 mL of Packard Ultra Gold\textsuperscript{TR} scintillation cocktail were then added to dried samples. Specific activity (SA) was measured in each incubated light sample at the end of the incubation; 100 µL sample was added to 200 µL phenylethylamine and 10 mL of UltraGold\textsuperscript{TR} scintillation cocktail was added. The radioactivity (disintegrations per minute) was measured in both types of samples using a Liquid Scintillation Analyzer (Tri-Carb 2910 TR).

The photosynthetic rate was calculated using the disintegration per minute (dpm), which measures the decay of electrons, i.e. when an electron disintegrates a flash of light is created. This dpm was multiplied by the DIC (pg C ml\textsuperscript{-1}) concentration and normalized. Specifically, the photosynthetic rate (pg C cell\textsuperscript{-1} h\textsuperscript{-1}) = \( \frac{dpm \times [\text{DIC}]}{SA \times h \times N} \) \( (3) \)

where SA (min\textsuperscript{-1} ml\textsuperscript{-1}) corresponds to the total activity in the sample, measuring the isotopes in both the water and the phytoplankton, across 3 h of incubation, \( h \) refers to the incubation times, and \( N \) to the number of cells in the vial (Eq. (2)).

For particulate organic C, N and P, samples were withdrawn and filtered onto GF/C glass fiber filters (47 mm), so that 7 - 10 x 10\textsuperscript{6} and 2.5 - 3 x 10\textsuperscript{5} cells were collected on the filters for \textit{T. amphioxeia} and \textit{M. rubrum}, respectively. The samples of \textit{T. amphioxeia} were filtered at low pressure below 3 Hg, to avoid washing out the biomass during filtration. For \textit{M. rubrum} gentle filtrate was achieved by hand pump due to their larger cell size. After filtration samples were packaged in aluminium and dried at 60 °C for 24 h and subsequently stored in the freezer at −20 °C until analysis. Prior to analysis, filters were cut using a cutting template with a scalpel into quarters. One quarter was folded into a 5 x 8 mm tin cup for CN analysis on a Flash 1112 EA Analyser (Interscience, Breda). The EA Analyser was calibrated using 3 standards, nicotinamide (22.94%N, 59.01%C), sulfanilamide (16.27%N, 41.84%C) and L-aspartic acid (10.52%N, 36.09%C). Phosphorus was determined by ashing another quarter of a filter in a pyrex tube for 30 min at 550 °C followed by autoclave digestion in 10 mL of 2.5% K\textsubscript{2}S\textsubscript{2}O\textsubscript{8} (m/v) for 20 min at 121 °C. The digest was then measured on an SAN++ continuous flow analyzer system (Skalar, Breda).

2.3. Statistics

Each combination of outcome (growth rate, cellular Chl a, cellular C content (pg C), C:N and C:P and species (\textit{M. rubrum}, \textit{T. acuta} and \textit{T. amphioxeia}) was analyzed separately. For \textit{M. rubrum}, analyses of growth rates were carried out both with and without the pH 6.8 treatment; for other outcomes pH 6.8 were not included. All pH levels were included for \textit{T. amphioxeia} and \textit{T. acuta}. Two statistical models were used. First, a two-way ANOVA with effects of pH and DIC (both as categorical variables) and their interaction was used to test for an interaction effect...
between pH and DIC, for linearity of the DIC effect, for an overall pH effect and an overall DIC effect. Tests for linearity were carried out because physiological rates are expected to follow saturation kinetics in the case of inorganic carbon and a dose-response curve in the case of direct pH effects. The overall effects were evaluated with marginal means, averaging over the other variable. Second, a linear regression with $p_{CO_2}$ as the only explanatory variables was used to assess the overall association between $p_{CO_2}$ and outcome. Holm’s method for multiple testing (Holm, 1979) was applied to the P-values for the different outcomes (for each type of hypothesis separately). Unadjusted P-values were reported, but significance was based on the Holm adjusted P-values with a significance level of 0.05. The analyses were performed using R.

3. Results

3.1. Experiment 1. The growth rate at different pH and DIC treatments

Unadjusted P-values are reported in Table 1, with asterisks denoting significance after Holm adjustment of the P-values (Holm, 1979). The adjustment was carried out for each type of hypothesis (each column with P-values) separately. Likewise, significance in the text below is based on the Holm adjusted P-values. Average growth rates of $T_.acuta$, $T_.amphioxeia$, and $M_.rubrum$ were in the range 0.37 - 0.71, and 0.59 - 0.82 d$^{-1}$ across all pH and DIC treatments, respectively. There was a significant difference between pH groups for both species (Table 1), but no overall difference between DIC treatments. Average growth rates of the kleptoplastidic ciliate, $M_.rubrum$ were in the range 0.31 - 0.62 d$^{-1}$ (Fig. 1e) in the pH treatments 7.1 - 8.0. In the pH 6.8 treatment $M_.rubrum$ ceased to survive at DIC levels of 6.5 and 13 mg C L$^{-1}$, and could not suppress its prey, $T_.amphioxeia$, and $M_.rubrum$ was overgrown. Therefore, two analyses were done for $M_.rubrum$, one including and one excluding pH 6.8. If the pH 6.8 treatment was left out, significant effects of pH were observed (Table 1), while there were no significant effects of DIC or interactions with pH. If the pH 6.8 treatment was included, then significant effects of both pH and DIC were observed.

The hypothesis of a linear DIC effect on growth rates was tested for all species (regression vs ANOVA) and was never rejected. The interaction between pH and DIC in two-way ANOVA was significant for $M_.rubrum$ when pH treatment 6.8 was included and for $T_.amphioxeia$, but not for either species without the pH 6.8 treatment. The growth rates of $T_.acuta$, $T_.amphioxeia$, and $M_.rubrum$ were also plotted as a function of $p_{CO_2}$ (Fig. 1b, d, f). A significant effect was observed in the cases of $M_.rubrum$ without the pH 6.8 treatment, but not in cases of $T_.acuta$, $T_.amphioxeia$ and $M_.rubrum$ if the pH 6.8 treatment was included.

3.2. Experiment 2. $T_.amphioxeia$ and $M_.rubrum$ - Cellular Chl a content, photosynthetic rates, and C:N:P stoichiometry at different pH and DIC treatments

The average cellular Chl a content in $T_.amphioxeia$ was in the range of 0.23 - 0.43 pg cell$^{-1}$ across the different pH and DIC treatments.
There was a significant overall difference between pH treatments for cellular Chl \( \text{a} \) content as well as for photosynthetic rates (Fig. 2c). There was no significant differences between the pH treatments in the response of Chl \( \text{a} \) contents and photosynthetic rates to DIC treatments. No significant differences were observed between the pH treatments in the response of Chl \( \text{a} \) contents and photosynthetic rates to DIC treatments.

### Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>pH/culture medium</th>
<th>Growth rate ((\text{d}^{-1}))</th>
<th>The pH effect on growth rate</th>
<th>pH effect on photosynthesis</th>
<th>Chl ( \text{a} ) content ((\text{pg chl} \text{a cell}^{-1}))</th>
<th>The pH effect on Chl ( \text{a} ) content</th>
<th>T ((\degree \text{C}))</th>
<th>I ((\mu \text{mol photons m}^{-2} \text{s}^{-1}))</th>
<th>Salinity</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. amphioxeia</td>
<td>K-1837</td>
<td>6 - 8.5 f/2</td>
<td>0.8 (pH: 6.6 - 8.5)</td>
<td>S-L at pH 6.4: (0.4 d^{-1})</td>
<td>0.23 - 0.33 S-NCR</td>
<td>5</td>
<td>20 - 50</td>
<td>30</td>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. amphioxeia</td>
<td>K-1837</td>
<td>6.8 - 8 f/20</td>
<td>0.59 - 0.82 S-NCR</td>
<td>S-NCR</td>
<td>0.23 - 0.33 S-NCR</td>
<td>5</td>
<td>20 - 50</td>
<td>30</td>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. amphioxeia</td>
<td>AND- A0710</td>
<td>6.5 - 8.6 L1</td>
<td>0.77 ± 0.10 NS</td>
<td>0.41</td>
<td>17.8 ± 6.6</td>
<td>100</td>
<td>35</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. amphioxeia</td>
<td>AND- A0710</td>
<td>6.5 - 8.6 (pH: 7.6) L1</td>
<td>0.85 ± 0.09 NS</td>
<td>0.24</td>
<td>17.8 ± 6.6</td>
<td>400</td>
<td>35</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R. marina</td>
<td>K-0435</td>
<td>7 - 8.5 f/2</td>
<td>0.55 - 0.6* NS</td>
<td>0.24</td>
<td>17.8 ± 6.6</td>
<td>400</td>
<td>35</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. cryophila</td>
<td>CCMP 2564</td>
<td>7.8 - 8.1 f/2</td>
<td>0.15 - 0.2* NS</td>
<td>N.S.D.</td>
<td>pH 7.8: 0.66</td>
<td>2</td>
<td>200</td>
<td>30</td>
<td>D</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ref. stands for reference: (A): Berge et al., 2010 (Denmark), (B): this study (Denmark), (C): Gaillard et al., 2020 (France), (D): Trimborn et al., 2019 (Antarctic). NS = No significant effect in the pH range studied, S-L = Significantly lower, S-NCR = Significant differences, but no consistent relationship, * = Estimated by graph.
changes in pCO₂ (Figs. 2b, d).

The average cellular Chl a content and photosynthetic rates in *M. rubrum* ranged between 6.8 - 23.6 pg cell⁻¹ and 19.1 - 61.2 pg C cell⁻¹ h⁻¹ across the different pH treatments, respectively (Fig. 3a, c). Significant differences were found between the pH and DIC treatments for the cellular Chl a content, and for pH effects on the photosynthetic rates (Table 1). However, no significant effects of DIC were observed on photosynthetic rates.

Significant differences were found between the pH treatments for the cellular carbon content, and the C:N and C:P ratios for both *T. amphioxeia* and *M. rubrum*, while significant differences between the DIC treatments only were found for carbon content in both species (Table 1, Fig. 4a,c, 5a,c, and 6a,c). Cellular carbon contents of both *T. amphioxeia*, and *M. rubrum* decreased in most cases with increasing DIC content of the water, while this was not the case for either the C:N or C:P ratios.

The assumption of a linear DIC effect on Chl a, rates of photosynthesis, cellular carbon content, and the C:N and C:P ratios for both *T. amphioxeia* and *M. rubrum*, while significant differences between the DIC treatments only were found for carbon content in both species (Table 1, Fig. 4a,c, 5a,c, and 6a,c). Cellular carbon contents of both *T. amphioxeia*, and *M. rubrum* decreased in most cases with increasing DIC content of the water, while this was not the case for either the C:N or C:P ratios.

A significant effect of pCO₂ was only observed in the cases of carbon content of *M. rubrum* without the pH 6.8 treatment. Highest carbon content was generally observed at the lowest pCO₂ at all pH levels (Figs. 4b, d).

4. Discussion

4.1. Are rates of photosynthesis and growth of *Teleaulax* spp. and marine cryptophytes generally affected by lowered pH and dissolved inorganic carbon?

Our results indicated significant pH effects on growth rates of the two *Teleaulax* species as well as on the rates of photosynthesis, cellular carbon and Chl a contents, and the C:N and C:P ratios in *T. amphioxeia*. However, responses of growth and photosynthetic rates to pH were not consistent in the range of pH 6.8 - 8, which suggests that these observed differences likely have resulted from experimental variation. Also, the DIC concentration had no significant effect on growth rates in *T. acuta* and *T. amphioxeia*, nor on the photosynthetic rate in *T. amphioxeia*. We could thus only partly confirm hypothesis 1, as our results gave no indications of negative effects of lowered DIC content on either growth or photosynthetic rates. Also, we found that the carbon content of *T. amphioxeia* increased with decreasing DIC level, which is opposite to our hypothesis 1. We could also not confirm hypothesis 3, as the C:N and C:P ratios of *T. amphioxeia* were not significantly affected by the DIC concentration.

Only few laboratory studies have investigated the effects of lowered pH on the ecophysiology of cryptophytes and only for a couple of cryptophyte species, *T. amphioxeia* and *Rhodomonas* sp. Our data on *T. amphioxeia* are in accordance with Berge et al. (2010), who investigated the same strain and found that the growth of *T. amphioxeia* was unaffected by acidification until pH was as low as 6.5. In their study, growth rates were reduced to half of the maximum growth rate at a pH of 6.3 and mortality rates were high (up to ~1.5 day⁻¹) at pH 6.0 (Table 2). Recently, a study by Gaillard et al. (2020) found similar results regarding the effects of the pH on growth rates for a Spanish strain of *T. amphioxeia* (AND-A0710), which was also largely unaffected in the pH range from 6.5 to 8.6 (Table 2).

A study on *Rhodomonas marina* demonstrated that the growth rate of this cryptophyte was not significantly affected by pH until it had reached pH 6.8 (Berge et al., 2010), and half of the maximum growth rate was still observed at pH of ~ 6.3. Net mortality was not observed until pH was lowered to ~ 5.9. Mesocosm experiments with natural plankton communities did not find any significant effects of an elevated pCO₂ treatment that corresponded to changes in pH from 8.13 to 7.82 with regard to either growth rate, cellular Chl a content, or the photosynthetic rates in the cryptophyte *Geminigera cryophila* (Table 2; Trimborn et al., 2019). Likewise, two mesocosm studies, which included cryptophytes at high pCO₂ (750 µatm), corresponding to pH 7.8, did not show effects on growth (Newbold et al., 2012 and Hopkins et al., 2010).

Thus, the available literature at present suggests that cryptophytes in general seem quite tolerant to lowered pH and lowered DIC. All cryptophytes have been shown to possess the form 1D RubisCo (Giordano et al., 2005). This form of RubisCo requires less energetic investment for CCMs under low CO₂ conditions (Giordano et al., 2005; Rickaby and Hubbard, 2019). Consequently, higher CO₂ levels and the potential down-regulation of these CCMs may have limited benefits to the cells, and possibly explain the lack of responses that have been observed in earlier studies with species relying more strongly on CCMs, such as cyanobacteria and dinoflagellates (Kranz et al., 2011; Eberlein et al., 2016; Van de Waal and Litchman, 2020). However, there may be many regulatory mechanisms not included in this study, and further work assessing CCMs and overall cellular metabolism in cryptophytes is needed to understand their resilience towards changes in pH and DIC.

4.2. Are rates of photosynthesis and growth of *M. rubrum* and other kleptoplastidic protists sensitive to lowered pH and dissolved inorganic carbon?

Previous studies with *M. rubrum* found similar rates of growth and photosynthesis as we found in this study in the pH range of 7.7 - 8.5 (Hansen and Fenchel, 2006; Smith and Hansen, 2007; Drumm et al., 2021). Our results showed significant pH effects on rates of growth and photosynthesis in *M. rubrum*. However, the responses to changes in pH (7.1 - 8.0) were not consistent (Fig. 1e), and the observed changes may thus have resulted from experimental variation. Concentrations of DIC in the range of 6.5 - 26 mg C.L⁻¹ did not affect rates of growth and photosynthesis in this pH range. The growth of this ciliate was, however, significantly reduced at pH 6.8 at lowered DIC concentrations (6.5 - 13 mg C.L⁻¹), suggesting an interactive effect where the response to the low pH depends on DIC concentrations. Thus, our results meet our hypothesis 2. However, since most coastal areas rarely experience pH below 7.1 (Carstensen et al., 2018; Carstensen and Duarte, 2018), our data indicate that these ciliates will very rarely be growth limited by low pH and/or DIC concentration.

Significant pH effects on the C:N:P stoichiometry were found in *M. rubrum* (Table 1). However, contrary to our hypothesis 3, the ciliate had higher carbon contents at low DIC concentrations, while the effects on C:N and C:P were not consistent across treatments. It is unclear why cellular carbon contents would accumulate under low DIC concentrations, or why cellular C:N and C:P ratios remained unaltered with elevated pCO₂. Similarly, there were no indications that increased CO₂ availability led to increased carbon contents and alteration of C:N:P stoichiometry; in fact, rather the opposite was found. These observations are not in line with the general response observed across phytoplankton species, which shows a general increase in C:N and C:P ratios with elevated pCO₂ (Velthuis et al., 2022). It is conceivable that because *M. rubrum* uses chloroplasts of *T. amphioxeia* with RubisCO type ID and therefore relies less on CCMs, it is also less sensitive to changes in pCO₂. However, this needs to be tested in dedicated experiments assessing CCM regulation in *M. rubrum* under a pCO₂ gradient.

The red *Mesodinium major/rubrum* species complex consists of eight different clades (clades A-H; Johnson et al., 2016). Our isolate belongs to “clade F”, which is common in brackish waters. To which extent other clades respond similarly to pH and DIC in the ranges studied here is unknown. Also, we were unable to find controlled laboratory studies of the effects of OA on other kleptoplastidic ciliates like *Strombidium* spp., *Laboea strobila* and *Tontonia* spp.
5. Conclusion and further perspectives

This study found no consistent changes in the ecophysiology of any of the cryptophytes and the kleptoplastidic ciliate *M. rubrum* when they were exposed to very low pH and DIC concentrations, except for *M. rubrum* at pH 6.8 and low DIC concentrations, indicating that they seem quite resilient towards OA, even in low salinity and low DIC systems like fjords and embayments. *M. rubrum* supplies chloroplasts to the diarthritic shellfish toxin (DSP) producers *Dinophysis* spp. Future studies on the consequences of OA for *Dinophysis* are required to assess the implications of the here observed lack of effects of OA on its prey, combined with potential direct OA effects. This may influence the occurrence of red tides and DSP events caused by these mixotrophic di- noflagellates. Most laboratory experiments on phytoplankton and OA have been carried out with monocultures of the species. These studies indicate a large interspecific variation among microalgae towards acidification. Nevertheless, *M. rubrum* and *Teleaulax* spp seem to be among the most tolerant species to acidification among published studies of phototrophic protists. Very few studies have been carried out on differential effects of pH and inorganic carbon, and thus new studies are required to separate effects of DIC and lowered pH on growth rates. However, many phytoplankton species are in fact mixotrophs, and it is currently unknown how this impacts the abilities of these organisms to cope with OA.

Author declaration

Christine Schultz Yde Eriksen, Melanie Desmaret Walli and Per Juel Hansen conceived and designed the experiments. Christine Schultz Yde Eriksen and Melanie Desmaret Walli performed the experiments, sampling and cell enumeration of the organisms, measurements of photosynthesis, and chlorophyll a. Adm. B. van De Waal and Nico Helmsing performed the analysis of the particulate organic carbon. Emma Ove Dahl and Helle Sørensen carried out the statistical analysis together with Per Juel Hansen. Per Juel Hansen supervised the experimental work. Christine Schultz Yde Eriksen, Melanie Desmaret Walli visualized the data and wrote the original draft of the manuscript. All contributed to the editing and review of the paper.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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References


