Short-term effects of winter warming and acidification on phytoplankton growth and mortality: more losers than winners in a temperate coastal lagoon

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Abstract Changes in temperature and CO_2 are typically associated with climate change, but they also act on shorter time scales, leading to alterations in phytoplankton physiology and community structure. Interactions among stressors may cause synergistic or antagonistic effects on phytoplankton dynamics. Therefore, the main goal of this work is to understand the short-term isolated and interactive effects of warming and high CO_2 on phytoplankton nutrient consumption, growth, production, and community structure in the Ria Formosa coastal lagoon (southern Portugal). We performed microcosm experiments with temperature and CO_2 manipulation, and dilution experiments under temperature increase, using winter phytoplankton assemblages. Phytoplankton responses were evaluated using inverted and epifluorescence microscopy. Overall, phytoplankton growth and microzooplankton grazing on phytoplankton decreased with warming. Negative antagonist interactions with CO_2 alleviated the negative effect of temperature on phytoplankton and cryptophytes. In contrast, higher temperature benefited smaller-sized phytoplankton, namely cyanobacteria and eukaryotic picophytoplankton. Diatom growth was not affected by temperature, probably due to nutrient limitation, but high CO_2 had a positive effect on diatoms, alleviating the effect of nutrient limitation. Results suggest that this winter phytoplankton assemblage is well acclimated to ambient conditions, and short-term increases in temperature are detrimental, but can be alleviated by high CO_2.

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Introduction

Phytoplankton are fundamental components of most aquatic communities and have been successfully employed as indicators of ecological status and environmental variability (Platt & Sathyendranath, 2008; Racault et al., 2012). Phytoplankton dynamics have been increasingly used for detecting and

forecasting climate change effects on aquatic ecosystems, particularly the long-term effects of increased temperature and carbon dioxide (Chavez et al., 2011; Dutkiewicz et al., 2013, 2015; Cloern et al., 2016; Henson et al., 2016, 2017). However, these climate change variables can also act on shorter time scales, affecting phytoplankton growth and mortality, both directly and indirectly. For instance, large diurnal fluctuations in pH, which can exceed 1 unit, have been observed in coastal waters (Nielsen et al., 2012; Cornwall et al., 2013). These changes are not related with ocean acidification, but rather with shifts in primary and secondary production, river runoff, and upwelling (Eriander et al., 2016 and references therein). Likewise, sea surface temperature may vary significantly on a daily basis (Stuart-Menteth et al., 2003), and diel amplitudes up to 3.5°C have been reported (Stramma et al., 1986). Thus, the study of short-term effects of environmental drivers is critical to understand basic physiological and ecological mechanisms that may modulate phytoplankton acclimation and adaptation to environmental variability.

The effects of high CO_2 on phytoplankton dynamics are species-specific and sometimes contradictory (Sobrino et al., 2008; Boyd et al., 2010; Meyer & Riebesell, 2015; Bach & Taucher, 2019). Overall, diffusion of CO_2 to the water decreases pH levels and leads to alterations in fundamental biogeochemical processes that depend on seawater CO_2 chemistry, potentially affecting marine biodiversity, particularly calcifying organisms (e.g. coccolithophores, Riebesell, 2004; Dutkiewicz et al., 2015). Increases in CO_2 may also enhance phytoplankton growth and productivity, due to a downregulation of carbon concentrating mechanisms which increase the energy available for other cellular processes (Giordano et al., 2005; Dutkiewicz et al., 2013). Changes in seawater temperature can have direct effects on phytoplankton metabolic rates and, within defined temperature ranges, cell division rates will increase with temperature and decrease above the upper temperature limit (Eppley, 1972; Collos et al., 2005; Daufresne et al., 2009; Sherman et al., 2016). A gradual shift towards smaller phytoplankton is also expected with ocean warming, driven by increased water column stratification and reduced nutrient availability (Agawin et al., 2000; Daufresne et al., 2009; Morán et al., 2010b; Sommer et al., 2015; Schmidt et al., 2020). In addition, heterotrophic processes, including metabolic and

grazing rates of zooplankton (Rose & Caron, 2007; Tsai et al., 2015, 2016), are usually considered more sensitive to temperature than autotrophic processes (López-Urrutia et al., 2006). In this context, the role of microzooplankton, mostly dominated by phagotrophic protists that are able to remove, on average, 62% of phytoplankton daily production (Schmoker et al., 2013), is particularly relevant. Temperature increases have been associated with higher microzooplankton growth rates and grazing rates upon phytoplankton (Aberle et al., 2007, 2012, 2015; O'Connor et al., 2009; Rose et al., 2009; Chen et al., 2012; Horn et al., 2020), eventually leading to a reduction in phytoplankton biomass. The impact of ocean warming on phytoplankton grazing is thus a key topic on climate change research, given that herbivory significantly affects phytoplankton biomass, the efficiency of the biological pump, and the resources available to higher trophic levels (Caron & Hutchins, 2013; Behrenfeld & Boss, 2014). Overall, the impact of warming on phytoplankton and their prevalent mechanisms (i.e. direct metabolic effects driven by warming, indirect effects linked with changes in stratification and nutrient supply, indirect effects driven by increased microzooplankton grazing) are expected to vary in time and space depending on light and nutrient conditions (Sommer et al., 2012; Lewandowska et al., 2014; Dutkiewicz et al., 2015).

Moreover, the occurrence of simultaneous changes in temperature, CO_2, and other phytoplankton drivers (e.g. nutrients, light, grazers) may show synergistic and/or antagonistic effects on phytoplankton, thus leading to variable responses of phytoplankton to CO_2 (Fu et al., 2012; Dutkiewicz et al., 2015; Liu et al., 2017) and temperature increases (O'Connor et al., 2009; Lewandowska et al., 2014). Addressing the interactions between high CO_2 and warming is particularly pertinent, as, overall, CO_2 may dampen the growth-enhancing effects of high temperature (Seifert et al., 2020), but the response direction highly depends on the functional group/species (Kroeker et al., 2013). Multi-stressor studies addressing the interactions between temperature and CO_2 have mostly used unialgal cultures (e.g. Fu et al., 2007, 2008; De Bodt et al., 2010; Torstensson et al., 2012) to assess effects on phytoplankton physiology. Other studies have analysed the interactive effects of temperature and CO_2 on natural phytoplankton assemblages or specific functional types, using a variety of

experimental approaches, including continuous cultures (Hare et al., 2007; Feng et al., 2009; Rose et al., 2009), semi-continuous cultures (Tatters et al., 2013; Hoppe et al., 2018), and natural microcosms (Coello-Camba et al., 2014; Holding et al., 2015; Maugendre et al., 2015), and incubation periods ranging between 1 day (Holding et al., 2015) and 30 + days (Keys et al., 2018).

The Ria Formosa coastal lagoon (Southern Portugal), located in an area extremely vulnerable to climate change (IPCC, 2014), is one of the most important confined marine ecosystems in Portugal, from biological and social–economical perspectives. Climate predictions for the southern Portuguese coast include a sea surface temperature increase of 3°C in the winter and 4°C in the summer by 2080–2100 (Santos et al., 2002). Moreover, a significant interannual winter warming trend (0.07–0.10°C yr^{-1}) since 1967, concurrent with a declining trend in phytoplankton biomass, were observed for this ecosystem (Barbosa, 2010). This declining trend could be indirectly related to the stimulatory effect of warming on phytoplankton herbivores, but as recognized by Barbosa (2010), dedicated experiments are needed to conclusively establish direct links between phytoplankton dynamics and climate change. Previous experiments addressing climate change impacts on phytoplankton in the Ria Formosa evaluated short-term interactive effects of high CO_2 and exposure to ultraviolet radiation (UVR) during winter (Domingues et al., 2014), and effects of CO_2, UVR, and nutrient enrichment during summer (Domingues et al., 2017b). However, no information on the effects of warming on phytoplankton and their dominant grazers is currently available for this climate-sensitive ecosystem.

In this context, this study aimed to understand the short-term isolated and combined effects of warming and high CO_2 on phytoplankton growth and mortality due to microzooplankton grazing in the Ria Formosa coastal lagoon during winter, a period commonly characterized by high nutrient availability (Domingues et al., 2017a) and low UVR (Machado, 2010). Our specific objectives were to evaluate: (a) the isolated and combined effects of warming and high CO_2 on phytoplankton net growth rate, composition, photosynthetic parameters, and nutrient consumption; and (b) the effects of temperature increase on phytoplankton group-specific instantaneous growth rates and microzooplankton grazing rates. To accomplish

these objectives, we used natural phytoplankton assemblages from the Ria Formosa coastal lagoon subjected to (a) a two-stressor microcosm experiment with temperature and CO_2 increase, and (b) a dilution experiment under in situ and increased temperature. Based on previous studies (Barbosa, 2010; Morán et al., 2010a; Domingues et al., 2014, 2017b; Sherman et al., 2016), we hypothesized that: (a) the effects of warming and high CO_2 on phytoplankton will vary among phytoplankton functional groups, with beneficial effects of temperature for smaller-sized groups, which can be counteracted by CO_2, and (b) warming will increase microzooplankton grazing on phytoplankton, particularly on picophytoplankton and nano-sized plastidic flagellates.

Materials and methods

Study site

The Ria Formosa coastal lagoon is a shallow multi-inlet euryhaline system, located on the southern coast of Portugal (Fig. 1). The lagoon has a total wetland area of 105 km^2, a mean depth of 2 m, and extends 55 km (E–W) and 6 km (N–S) at its widest point (Andrade et al., 2004). Tides are semidiurnal and mesotidal, with tidal amplitudes ranging between 1.3 and 3 m during neap and spring tides, respectively (Newton et al., 2003). The water column is well mixed, with no persistent or widespread haline or thermal stratification (Newton & Mudge, 2003). Short-term variations in water temperature and pH are not significant in the Ria Formosa (Cravo et al., 2020). Water temperature variability reflects the seasonality of solar radiation, with lower values in January and February ($\sim$ 10°C), and higher values during summer months (15–27°C). pH values follow the inverse pattern, with lower values in the summer (7.75) and higher in the autumn/winter (8.30); pH values mirror the variability in dissolved oxygen concentrations and lower values are associated with coastal upwelling events (Cravo et al., 2020).

The Ria Formosa is located in a Mediterranean climate area, with hot and dry summers, and moderate winters. The coastal region adjoining the Ria Formosa is affected by regular upwelling events that extend approximately 6 km upstream the lagoon inlets (Cravo et al., 2014). The lagoon is a breeding and
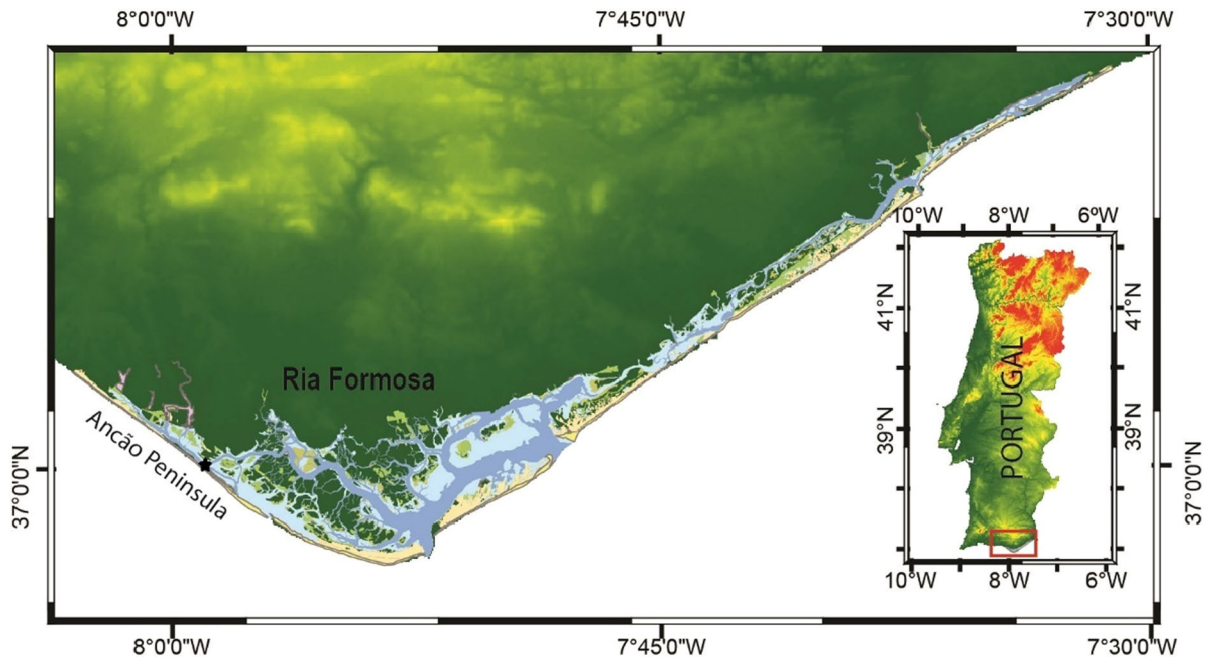
Fig. 1 Location of the Ria Formosa coastal lagoon and sampling site (black star) (map kindly provided by Dr. Susana Costas, CIMA-UAlg)

feeding ground for fish and birds, and supports a wide range of human activities, including tourism, fisheries, and shellfish farming. Due to its ecological importance, the Ria Formosa lagoon and adjacent habitat were designated a National Natural Park in 1987, part of Natura 2000 European conservation network. The Ria is a Ramsar wetland and it is included in the Special Bird Protection Area.

Experimental strategy

Two experiments were carried out with water samples collected in the Ria Formosa coastal lagoon, under typical winter conditions (February 2012). For both experiments, sub-surface water samples were collected using 10 l polycarbonate bottles at an inner lagoon location (Fig. 1) at low tide, to minimize the influence of adjacent coastal waters. Water temperature and photosynthetically active radiation in the mixed layer were measured with a YSI 30 probe and a LI-COR LI-193 spherical quantum sensor, respectively. Water samples were collected for the determination of inorganic macronutrient concentrations, chlorophyll-*a* concentration, and phytoplankton composition and abundance, at the beginning of the

experiments. Samples were not screened prior to incubation, to avoid damaging delicate microzooplankton (Calbet et al., 2011b; Grinienė et al., 2016) and modifying initial phytoplankton composition (Nogueira et al., 2014). All material used in the experiments and laboratorial analyses was previously washed with HCl 10% and thoroughly rinsed with deionised water.

Experiment 1: effects of warming and high CO_2 on nutrient consumption, phytoplankton growth and composition, and photosynthetic parameters

The first experiment tested the effects of warming and high CO_2 on the net growth rate and photosynthetic parameters of winter phytoplankton assemblages. Water samples were incubated under two different temperatures: (a) in situ temperature ($T_{is} = 10°C$), and (b) in situ + 3°C ($T_{is+3} = 13°C$). The use of a 3°C increase in temperature was based on model predictions for southern coast of Portugal by 2080–2100 (Santos et al., 2002). Due to logistical reasons, treatments subjected to increased temperature were conducted 2 days after treatments subjected to ambient temperature. For each temperature, ambient CO_2,

and high CO_2 treatments were prepared. Carbonate (CO_3^{2-}, as Na_2CO_3), bicarbonate (HCO_3^{-}, as $NaHCO_3$) and hydrochloric acid (HCl 0.01 N) were added to increase CO_2 partial pressure (pCO_2) and dissolved inorganic carbon (DIC) to levels expected for the year 2100, according to Gattuso et al. (2010). pCO_2 at the beginning and end of the incubation period was estimated with the Seacarb package for R (R Core Team, 2020). Alkalinity, determined by titration (Parsons et al. 1984), and pH were used as input variables. CO_2 partial pressure at the beginning of incubation was 389.7 ± 77.0 µatm and 712.3 ± 82.8 µatm in the low and high CO_2 treatments, respectively, corresponding to pH values of 8.1 ± 0.1 and 7.9 ± 0.0. After 2-day incubation, pCO_2 and pH were 379.4 ± 15.5 µatm and 8.1 ± 0.0 in the low CO_2 treatments and 719.0 ± 59.2 µatm and 7.9 ± 0.0 in the high CO_2 treatments.

Four different experimental treatments, established in triplicate 2 l polycarbonate bottles, were used to test the effects of warming and CO_2: "ambient" (in situ temperature, ambient CO_2 levels), " + CO_2" (in situ temperature, high CO_2), " + T" (increased temperature, ambient CO_2) and " + T + CO_2" (increased temperature, high CO_2). The bottles were incubated in a plant growth chamber (Phytoclima S600) for 48 h, under an ambient light:dark cycle (11 h:13 h), and exposed to photosynthetically active radiation (PAR) intensity of approx. 200 µmol photons m^{-2} s^{-1} (equivalent to mean PAR intensity in the mixed layer, estimated according to Domingues et al., 2017a). Bottles were manually homogenized twice daily to avoid settlement of non-motile cells. The experimental treatments were initially prepared in 10 l polycarbonate bottles, from which initial samples were taken; at the end of incubation, final samples were taken from each bottle. Initial and final water samples were used for the determination of pH and alkalinity, concentration of dissolved inorganic macronutrients, chlorophyll-a concentration, phytoplankton composition and abundance, and photosynthetic parameters. Phytoplankton net growth rates (r, d^{-1}) for the whole assemblage or specific functional types were estimated according to Eq. 1, assuming exponential growth:

$$r = \frac{\ln N_t - \ln N_0}{\Delta t} \tag{1}$$

where N_t and N_0 represent chlorophyll-a concentration or phytoplankton abundance at the end and beginning of incubation, respectively ($\Delta t = 2$ days). Nutrient net consumption rates or, more accurately, nutrient disappearance rates, that integrate not only nutrient uptake (inward transport through the cell membrane), but also excretion and regeneration (Domingues et al., 2011a), were estimated as the slope of a linear or exponential function adjusted to nutrient concentration over the incubation period.

Experiment 2: effects of temperature increase on phytoplankton growth, and mortality

The second set of experiments tested the effects of temperature on phytoplankton instantaneous growth rates and mortality rates due to microzooplankton herbivory, using the dilution technique (Landry & Hassett, 1982); the water samples were not prescreened, given that this may significantly alter the initial phytoplankton assemblage (Nogueira et al., 2014). A series of four different dilutions of whole seawater sample were prepared using seawater filtered through glass fibre filters (GF/F, Whatman, particle retention > 0.7 µm). Considering the dilution factor as the proportion of unfiltered seawater sample in each dilution treatment, the following dilutions were prepared: 0.125, 0.25, 0.50, and 1 (undiluted water). In order to avoid differences in nutrient concentration among dilutions, which could lead to the violation of method assumptions, all treatments were enriched with inorganic macronutrients, including silicate (Calbet & Saiz, 2018), at saturating concentrations (+ 50 µM of N and Si, added as nitrate and silicate, respectively, and + 2.5 µM of P, added as phosphate). Additionally, an experimental treatment with undiluted water without nutrient additions (DIL1) was also prepared to account for the potential effects of nutrient addition on the estimation of in situ phytoplankton instantaneous growth rates.

The five experimental treatments (dilutions) were prepared in 10 l polycarbonate bottles, from which the initial samples for the determination of in vivo fluorescence of chlorophyll-a, and phytoplankton composition and abundance were taken. Then, duplicate 1 l polycarbonate bottles were filled, with no headspace, with the respective dilution and incubated for 48 h inside a plant growth chamber, under

simulated in situ conditions (ambient light:dark cycle = 11 h:13 h; and PAR intensity of approx. 200 μmol photons m^{-2} s^{-1}, equivalent to mean PAR intensity in the mixed layer) and controlled temperature. Bottles were manually homogenized twice a day to avoid settlement of non-motile cells. Experiments were carried out under two different temperatures: (a) in situ temperature ($T_{is} = 13°C$), and (b) in situ + 3°C ($T_{is+3} = 16°C$). At the end of incubation, aliquots were taken from all experimental treatments for the determination of the above-referenced variables.

For each dilution, net phytoplankton growth rates (r, d^{-1}) during the incubation period (Δt) were calculated according to Eq. 1, using in vivo fluorescence of chlorophyll-a for the whole phytoplankton assemblage and cell abundance for specific phytoplankton groups, at the beginning (N_0) and end (N_t) of the incubation, assuming exponential growth. Phytoplankton net growth rates in nutrient-enriched dilutions were then regressed against the exact dilution factor (calculated as the quotient between in vivo fluorescence in each dilution and in vivo fluorescence in undiluted water), and phytoplankton potential instantaneous growth rates (μ_0) and microzooplankton grazing rates (g) were estimated as the y-intercept and the slope (when negative, absolute value is considered; for other situations, see below) of the linear regression line, respectively (Landry & Hassett, 1982). In situ instantaneous growth rate of phytoplankton (μ) was estimated according to Eq. 2 (Calbet et al., 2011a):

$$\mu = NGR.DIL1 + g \tag{2}$$

where NGR.DIL1 represents phytoplankton net growth rate in undiluted samples without added nutrients (DIL1). Dilution experiments were also used to assess phytoplankton potential nutrient limitation, by comparing phytoplankton net growth rates between nutrient-enriched and nutrient-unenriched undiluted treatments (NGR.DIL1 + and NGR.DIL1, respectively). Standard errors (SE) for μ and NGR were estimated using the corresponding error propagation equations (see Calbet et al., 2011a; http://julianibus.de/physik/propagation-of-uncertainty).

Analysis of variance (ANOVA) on the residuals of the linear regression was used to test whether the dilution plots significantly deviated from the linearity assumption of the dilution technique (Worden & Binder, 2003; Morison & Menden-Deuer, 2017). For linear plots with statistically non-significant slopes (i.e. not significantly different from zero), grazing rate was assumed zero, and phytoplankton potential instantaneous growth rate was estimated as the average value of the net growth rates of all nutrient-amended dilution treatments (Chen et al., 2009; Twiss & Smith, 2012; Grinienė et al., 2016; Morison & Menden-Deuer, 2017; Menden-Deuer et al., 2018; Jiang et al., 2021).

For dilution plots that showed significant deviations from linearity, mostly associated with saturated grazing (Gallegos, 1989; Dolan et al., 2000; Teixeira & Figueiras, 2009), data were also tested for non-linearity by polynomial regression analyses. When significant non-linearity was detected ($P < 0.05$, for the coefficient of quadratic or higher terms in actual dilution factor; (Nejstgaard et al., 1997; Suzuki et al., 2002)), phytoplankton potential instantaneous growth rate (μ_0) was estimated as the regression intercept of the linear (non-saturation range) portion of the dilution plot (generally dilution factor ≤ 0.5), and the microzooplankton grazing rate (g) was estimated as $\mu_0 -$ NGR.DIL1 + . This modified version of the three-point method (Gallegos, 1989) is widely used for analyses of non-linear dilution plots (Strom et al., 2001, 2007; Teixeira & Figueiras, 2009; Teixeira et al., 2011, 2012, 2014; Dix & Hanisak, 2015).

Although dilution is the most commonly used technique for measuring microzooplankton herbivory, the linearity of the dilution plots has been frequently compromised, namely in eutrophic ecosystems, therefore requiring a cautious interpretation (Calbet & Saiz, 2013; Dix & Hanisak, 2015; Horn et al., 2020). Significant negative slopes (one-tailed t-test, $P < 0.05$) for the whole or part (e.g. L-shaped, V-shaped, and inverted V-shaped dilution plots) of each dilution series were interpreted as a significant grazer effect on phytoplankton. In cases where significant negative slopes were observed, the percentage of particulate net phytoplankton production daily removed by microzooplankton (% pNPP) was estimated as ($g/\mu \times 100$) (Calbet et al., 2011a).

Inorganic macronutrients

Samples for determination of inorganic macronutrient concentrations were filtered through 0.2 μm cellulose acetate filters. Soluble reactive phosphorus (SRP), dissolved silicate (DSi), and ammonium (NH_4^+) were

analysed immediately after sampling, using the spectrophotometric methods described by Grasshoff et al. (1999) on a spectrophotometer Hitachi U-2000. Samples for nitrate (NO_3^-) and nitrite (NO_2^-) were frozen (− 20°C) until analysis on a Skalar SAN + segmented flow analyser. Dissolved inorganic nitrogen (DIN) concentration was calculated as the sum of NO_3^-, NO_2^-, and NH_4^+ concentrations.

Phytoplankton biomass, abundance, and composition

Chlorophyll-*a* concentration, used as a proxy for phytoplankton biomass, was determined spectrophotometrically after sample filtration through glass fibre filters (GF/F, Whatman, particle retention > 0.7 μm) and overnight extraction with acetone 90% (Parsons et al., 1984). For dilution experiments, chlorophyll-*a* in vivo fluorescence was determined using a Turner Designs 10-AU fluorometer.

Phytoplankton composition and abundance were determined using epifluorescence (Haas, 1982) and inverted microscopies (Utermöhl, 1958). Samples for pico- (< 2 μm) and nanophytoplankton (2–20 μm) analysis were preserved with glutardialdehyde (final concentration 2%) immediately after collection, stained with proflavine, and filtered (< 100 mm Hg) onto black polycarbonate membrane filters (Whatman, nominal pore diameter = 0.45 μm). Preparations were made within 24 h of sampling using glass slides and non-fluorescent immersion oil (Cargille type A), and then frozen (− 20°C) until analysis under dark conditions, to minimize loss of fluorescence. Observation was made at 787.5 × magnification using a Leica DM LB epifluorescence microscope. Samples for microphytoplankton (> 20 μm) analysis were preserved with acid Lugol's solution (final concentration approx. 0.003%) immediately after collection. Acid Lugol's was chosen given that it is more appropriate than neutral Lugol's in preserving microflagellates and diatoms (Williams et al., 2016), which are major components of phytoplankton in the Ria Formosa (Barbosa, 2010). Aliquots of 25–50 ml were settled in sedimentation chambers and observed at 400 × magnification using a Zeiss Axio Observer A1 inverted microscope. Cells were identified, whenever possible, to the genus or species level. For both methods, a minimum of 50 random visual fields and at least 400 cells in total, with 50 cells of the most common genus, were enumerated. Assuming that phytoplankton cells were randomly distributed, counting precision was ± 10% (Venrick, 1978). Phytoplankton taxonomic classification followed the online database AlgaeBase (Guiry & Guiry, 2021).

Phytoplankton primary production and photosynthetic parameters

Primary production and photosynthetic parameters were determined using the Steeman-Nielsen (1952) method. 50 ml aliquots of each experimental treatment were collected at the end of incubation into polycarbonate flasks and 100 μl (2 μCi) of ^{14}C-HCO_3^- were added to each flask. The flasks were incubated in a plant growth chamber, for 2 h, under different PAR intensities (0, 47, 113, 230, and 365 μmol photons m^{-2} s^{-1}), provided by Philips Cool White fluorescent lamps. Primary production was calculated according to Eq. 3:

$$PP = \frac{(R_s - R_b) \times D \times W}{R \times \Delta t} \qquad (3)$$

where PP is phytoplankton primary production (mg C L^{-1} h^{-1}), R_s (dpm) is the activity in the sample, R_b (dpm) is the mean activity of the dark flasks, D is the isotopic discrimination (= 1.05), W (mg C L^{-1}) is the amount of dissolved inorganic carbon in the sample, R (dpm) is the total activity of the ^{14}C added to each flask, and Δt is the incubation period (two hours). The concentration of dissolved inorganic carbon was analysed after acidification with HCl (Parsons et al., 1984).

For each experimental treatment and each light level, primary production (PP, mg C L^{-1} h^{-1}) was converted to biomass-specific primary production (P^B = PP/Chl*a*, mg C (mg Chl*a*)$^{-1}$ h^{-1}). Photosynthetic parameters were estimated according to Eilers & Peeters (1988) model, through non-linear fitting of Eq. 4, using the "nls" function available in R (R Core Team, 2020):

$$P^B = \frac{E}{aE^2 + bE + c} \qquad (4)$$

where P^B is biomass-specific primary production (mgC mgChl*a*$^{-1}$ h^{-1}) and E is PAR irradiance during incubation (μmol photons m^{-2} s^{-1}). Coefficients a, b, and c were used to estimate maximal biomass-specific production rate (P^B_{max}, mgC mgChl*a*$^{-1}$ h^{-1}), optimal

light intensity (E_{opt}, µmol photons m^{-2} s^{-1}), and initial slope of the *P–E* curve, which represents photosynthetic efficiency (α, mg C (mg Chl*a*)$^{-1}$ h^{-1} (µmol photons m^{-2} s^{-1})$^{-1}$), according to Eqs. 5, 6, and 7, respectively:

$$P_{max}^{B} = \frac{1}{b + 2\sqrt{ac}} \quad (5)$$

$$E_{opt} = \sqrt{\frac{c}{a}} \quad (6)$$

$$\alpha = \frac{1}{c} \quad (7)$$

Data analyses

Data normality and homogeneity of variances were tested using Shapiro–Wilk and Levene's tests, respectively. The isolated and combined effects of warming and high CO_2 on nutrient net consumption rates and phytoplankton net growth rates, for the phytoplankton assemblage and specific functional types, were assessed using a two-way analysis of variance (ANOVA), with replication. Effect sizes were assessed by estimating omega-squared statistics, which indicates the percentage of variation in the dependent variable attributable to the independent variable (Olejnik & Algina, 2003; Albers & Lakens, 2018). Differences in phytoplankton photosynthetic parameters and instantaneous growth rates, and microzooplankton grazing rates between in situ and increased temperature were evaluated using one-tailed *t*-test or Mann–Whitney test, depending on data normality. All statistical analyses were considered at a 0.05 significance level. Data analyses were performed with IBM SPSS® Statistics v.22 software.

Results

Environmental setting

Experiment 1 was used to evaluate the response of a natural plankton assemblage, collected under typical winter conditions (early February 2012, water temperature = 10°C) in the Ria Formosa coastal lagoon, to controlled increases in water temperature and carbon dioxide content, based on four different

experimental treatments: ambient (control), + CO_2, + T, and + T + CO_2. Initial concentrations of dissolved inorganic macronutrients were 0.95 ± 0.18 µM for DIN, 0.18 ± 0.05 µM for SRP, and 3.41 ± 1.38 µM for DSi. Initial chlorophyll-*a* concentration (Chl*a*) and total phytoplankton abundance varied between 1.11 and 3.88 µg L^{-1} and 5.26 × 10^6–5.36 × 10^6 cells L^{-1}, respectively. In terms of abundance, phytoplankton community was dominated by *Synechococcus*-like picocyanobacteria (85.1%), with minor contributions of cryptophytes (14.6%) and diatoms (0.3%).

Experiment 2 evaluated the effects of temperature (+ 3°C) on phytoplankton growth and mortality using a natural plankton assemblage collected in late February 2012 (water temperature = 13°C) in the Ria Formosa coastal lagoon. Initial nutrient concentrations were 2.75 ± 2.48 µM DIN, 0.09 ± 0.02 µM DRP and 4.69 ± 1.38 µM SRP. Initial Chl*a* concentration in these experiments was 3.0 ± 0.2 µg L^{-1} and total phytoplankton abundance was 3.34 × 10^6 ± 5.17 × 10^5 cells L^{-1}. Phytoplankton community was dominated, in terms of abundance, by eukaryotic picophytoplankton (38%) and cryptophytes (38%). Other autotrophic nanoflagellates (11%), cyanobacteria (5%), and diatoms (7%) dominated by the colonial centric genus *Chaetoceros*, were also present in the initial samples. Euglenophytes and dinoflagellates showed minor contributions to total abundance (1%). Initial abundance of phagotrophic protists was 3.90 × 10^5 ± 5.91 × 10^4 cells L^{-1} and the assemblage was mainly composed by aplastidic nanoflagellates (96%) and ciliates (4%).

Effects of warming and high CO_2 on nutrient consumption, phytoplankton net growth rate and composition, and photosynthetic parameters

Except for nitrate (Fig. 2A), temperature increase significantly affected net consumption rates of all nutrients (dissolved silicate, dissolved reactive phosphorus, and ammonium), in relation to the control (Fig. 2, Table 1). Under increased temperature, consumption rates of NH$_4^+$ (F(1,8) = 28.155; P = 0.001; ω^2 = 0.70; Fig. 3B) and DSi (F(1,7) = 114.005; P < 0.001; ω^2 = 0.71; Fig. 2D) were significantly higher, but SRP consumption (F(1,8) = 18.778; P = 0.003; ω^2 = 0.75; Fig. 2C) was significantly lower. DSi consumption was also significantly higher
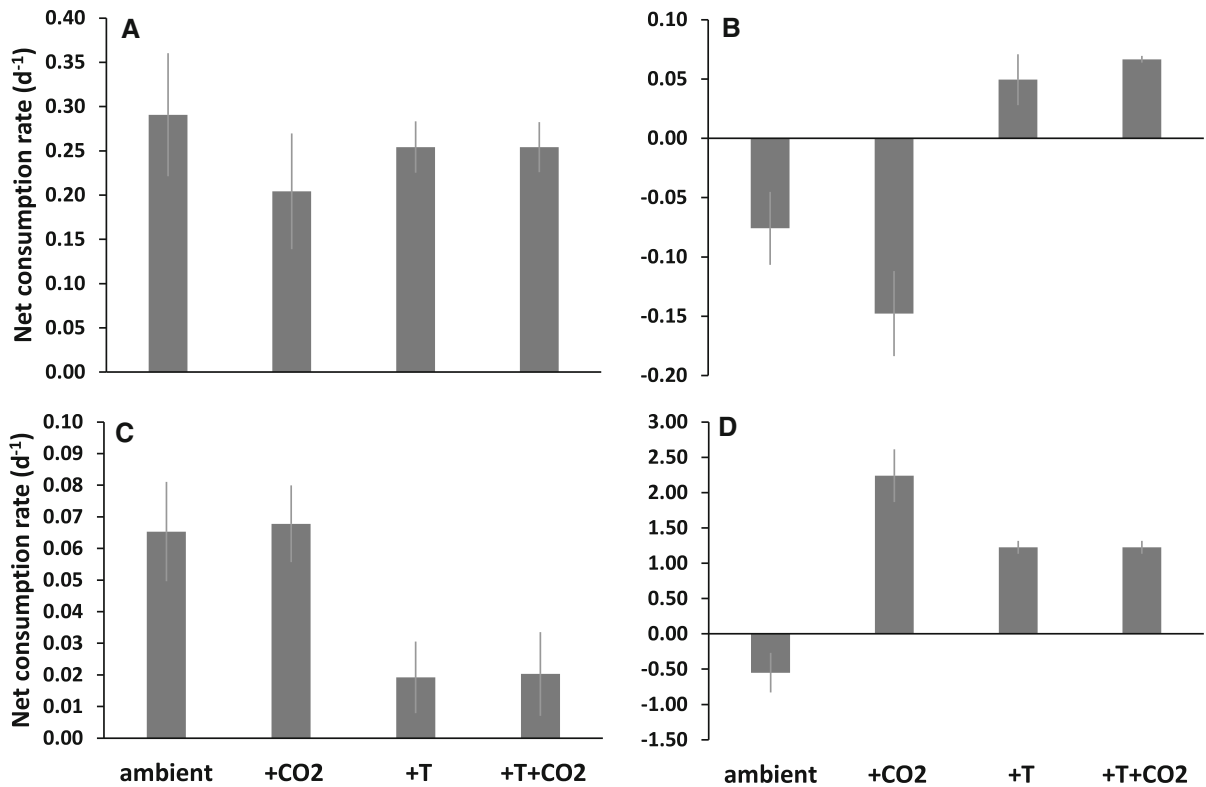
Fig. 2 Mean consumption rates (μM d^{-1}) of (**A**) nitrate, (**B**) ammonium, (**C**) phosphate, and (**D**) silicate in the temperature and CO_2 enrichment experiments (experiment 1). Vertical lines represent $\pm$ 1 standard error

Table 1 P-values (P) and omega-squared values (ω^2) obtained with two-way ANOVA for isolated and combined effects of increased temperature and CO_2 on phytoplankton community and specific net growth rates, and nutrient consumption rates for experiment 1, considered at a significance level of 0.05

|	T		CO_2		T*CO_2	
	P	ω^2	P	ω^2	P	ω^2
NIT	n.s	− .04	n.s	.21	n.s	.21
AMM	.001	.70	n.s	− .02	n.s	.02
P	.003	.75	n.s	.00	n.s	.00
Si	.000	.71	.014	.06	.014	.06
Community	.001	.45	n.s	− .02	.003	.33
Diatoms	n.s	− .04	.024	.32	n.s	.17
Cryptophytes	.000	.73	.007	.06	.000	.30
Cyanobacteria	.011	.60	n.s	− .05	n.s	− .05

n.s. not significant (see also Figs. 3 and 4)

*NIT* nitrate, *AMM* ammonium, *P* dissolved reactive phosphorus, *Si* dissolved silica

under high CO_2 (F(1,7) = 10.710; P = 0.014; ω^2 = 0.06), but the effect size was small. Significant (F(1,7) = 10.710; P = 0.014; ω^2 = 0.06) interactive effects between CO_2 and temperature increases on DSi consumption rates were also detected, but the magnitude of the interaction was low (Table 1).

Overall, phytoplankton abundance decreased in all experimental treatments during the 2-day incubation period, and changes in phytoplankton composition among experimental treatments were also detected. The relative contribution of cyanobacteria and diatoms to total phytoplankton abundance increased in all experimental treatments in relation to the control (between 111–146%, and 199–271%, respectively). Conversely, the relative contribution of cryptophytes decreased, for all the experimental treatments (58–89%), in relation to the control.

Significant responses of the whole phytoplankton assemblage to warming and high CO_2 were observed, in relation to the control treatment (Fig. 3, Table 1). Phytoplankton net growth rates, based on Chl*a*, were
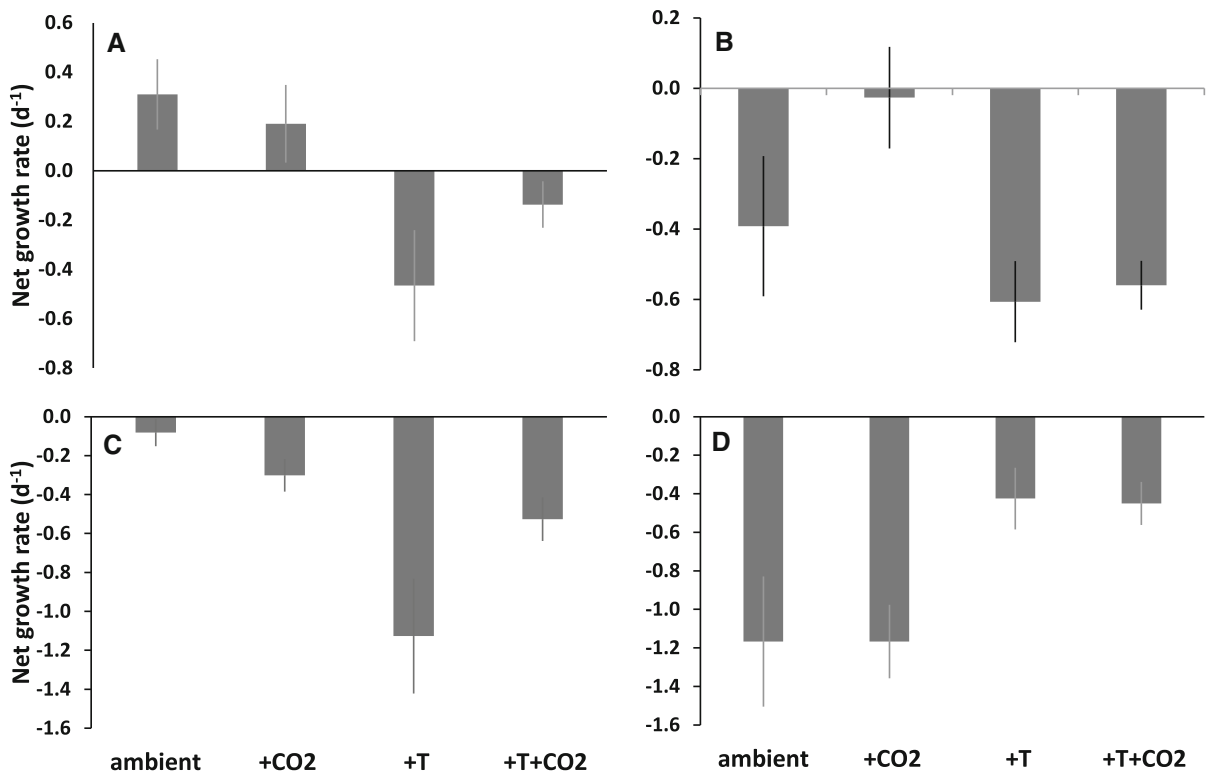
Fig. 3 Net growth rates (d^{-1}) of (**A**) phytoplankton community, (**B**) diatoms, (**C**) cryptophytes, and (**D**) cyanobacteria in the temperature and CO_2 enrichment experiments (experiment 1). Vertical lines represent ± 1 standard error
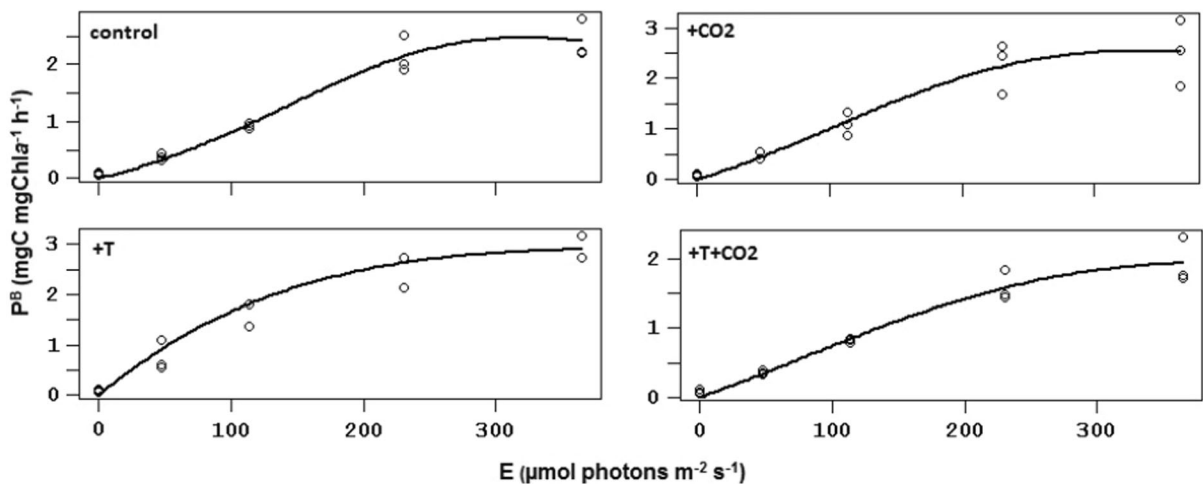
Fig. 4 Photosynthesis-irradiance curves, determined using ^{14}C incubations, in the temperature and CO_2 enrichment experiments (experiment 1), depicting biomass-specific production (P^B, mgC mgChla^{-1} h^{-1}) as a function of light (PAR) intensity (E, μmol photons m^{-2} s^{-1}). Curve fitting was based on the non-linear regression model of Eilers & Peeters (1988) (see Eq. 3)

negatively affected by warming ($F_{(1,8)} = 23.187$; $P = 0.001$; $\omega^2 = 0.45$), but were not affected by high CO_2 (p > 0.05). Significant negative interactive

effects of high CO_2 and warming were detected ($F_{(1,8)} = 17.489$; $P = 0.003$; $\omega^2 = 0.33$) (Table 1, Fig. 3A).

Diatom net growth rates, negative in all treatments, showed no significant responses to warming, but were significantly higher under high CO_2 (although still negative), in relation to the control ($F_{(1,8)} = 7.699$; $P = 0.024$; $\omega^2 = 0.32$) (Fig. 3B, Table 1). No interactions between temperature and CO_2 were detected. Cryptophyte net growth rates were negatively affected by both warming ($F_{(1,7)} = 157.284$; $P < 0.001$; $\omega^2 = 0.73$) and high CO_2 levels ($F_{(1,7)} = 14.053$; $P = 0.007$; $\omega^2 = 0.06$), in relation to the control (Fig. 3C, Table 2). Significant interactive effects of high CO_2 and warming on cryptophyte growth rate were also detected ($F_{(1,7)} = 65.337$; $P < 0.001$; $\omega^2 = 0.30$), leading to lower growth rates in relation to the control. Net growth rates of cyanobacteria were positively affected by warming ($F_{(1,6)} = 13.120$; $P = 0.011$; $\omega^2 = 0.60$), but high CO_2 levels showed no significant effects (Fig. 3D, Table 2).

Phytoplankton photosynthetic parameters, derived from photosynthesis-irradiance (PE) curves (Fig. 4, Table 2), showed only a few significant changes associated with temperature and CO_2. Photosynthetic efficiency (0.00574–0.02190 mgC mgChla^{-1} h^{-1} (μmol photons m^{-2} s^{-1})$^{-1}$) was significantly higher ($P < 0.05$) for isolated increases in temperature, in relation to the control, showing a 3.8-fold increase. In the case of maximum biomass-specific production rate, P_{max}^B (1.95–2.91 mgC mgChla^{-1} h^{-1}), a significant decline ($P < 0.01$) was associated with warming and high CO_2 ($+ T + CO_2$), in relation to the control.

Effects of warming on phytoplankton instantaneous growth and microzooplankton grazing rates

Dilution experiments were used to evaluate the effects of warming on phytoplankton potential (μ_o) and in situ (μ) instantaneous growth rates, and microzooplankton grazing rates (g) and impact (%pNPP) and detect potential nutrient limitation of phytoplankton growth. Mean values of μ_0, μ, g, and %pNPP, for the whole assemblage or specific phytoplankton functional groups, respective standard errors ($\pm$ SE), and relevant statistical information are presented in Table 3 (see Fig. 5 for dilution plots). The comparison of phytoplankton net growth rates in undiluted samples with and without added nutrients revealed a significant growth enhancement for diatoms, cryptophytes, and the whole phytoplankton assemblage, for both temperature levels (ambient and $+ 3°C$), and for other plastidic nanoflagellates and eukaryotic picophytoplankton under increased temperature, indicative of nutrient limitation. No apparent effects of nutrient addition on net growth rates of cyanobacteria were detected (see Fig. 5, Table 3). Mean values for μ, g, and grazing impact on daily phytoplankton production ranged between 0.132–0.913 d^{-1}, 0.069–0.876 d^{-1}, and 0–142.6%, respectively, varying according to the phytoplankton group considered and temperature level.

Overall, the effects of temperature increase were more marked on phytoplankton growth rates than on microzooplankton grazing rates. At the level of the whole phytoplankton assemblage (Fig. 5A), temperature increase induced a significant decline in both μ_0

Table 2 Photosynthetic parameters (± 1 standard error) obtained for each experimental treatment (experiment 1) through non-linear fitting of Eilers & Peeters (1988) PE model, where P_{max}^B is the maximal biomass-specific production rate (mgC mgChla^{-1} h^{-1}), I_{opt} is the optimal light intensity (μmol photons m^{-2} s^{-1}) and c is 1/α, with α the photosynthetic efficiency (mgC mgChla^{-1} h^{-1} (μmol photons m^{-2} s^{-1})$^{-1}$)

	P_{max}^B	I_{opt}	α
Ambient	2.47 ± 0.10	322 ± 25	0.00574 ± 0.00123
+ CO_2	2.54 ± 0.17	336 ± 69	0.00817 ± 0.00300
+ T	2.91 ± 0.38	401 ± 303	0.02190 ± 0.00964
+ T + CO_2	1.95 ± 0.15	408 ± 110	0.00685 ± 0.00179

Table 3 Information on mean values ($\pm$ 1 standard error, SE) of phytoplankton growth and mortality, for the whole phytoplankton assemblage and specific phytoplankton groups, derived from dilution experiments undertaken using in situ temperature (T_{is}) and increased temperature (T_{is+3} = in situ temperature $+ 3°C$)

		NGR.DIL1	NGR.DIL1 +	μ_0	μ	g	%pNPP	R^2	P-value	n
Phytoplankton (chlorophyll-a)	Tis	0.063 ± 0.043	$0.445 \pm 0.021^{\#\#}$	$\mathbf{0.848 \pm 0.070^{**}}$	$\mathbf{0.520 \pm 0.121^{*}}$	$\mathbf{0.457 \pm 0.113^{*}}$	87.9	0.731	0.007	8
	Tis+3	-0.044 ± 0.033	$0.330 \pm 0.035^{\#\#}$	0.504 ± 0.038	0.132 ± 0.072	0.176 ± 0.064	133.3	0.555	0.034	8
Planktonic diatoms	Tis	0.004 ± 0.012	$0.466 \pm 0.066^{\#\#}$	1.288 ± 0.157	0.880 ± 0.223	0.876 ± 0.222	99.5	0.795	0.017	6 (b)
	Tis+3	0.142 ± 0.063	$0.826 \pm 0.010^{\#\#}$	1.392 ± 0.138	0.714 ± 0.213	0.570 ± 0.203	79.8	0.663	0.049	6 (b)
Cryptophytes	Tis	-0.07 ± 0.0006	$0.057 \pm 0.038^{\#}$	$\mathbf{0.868 \pm 0.129^{**}}$	0.742 ± 0.155	0.812 ± 0.155	109.4	0.932	0.035	4 (b)
	Tis+3	-0.161 ± 0.029	$-0.054 \pm 0.017^{\#}$	0.474 ± 0.039	0.378 ± 0.072	0.539 ± 0.066	142.6	0.918	0.0002	8
Other plastidic nanoflagellates	Tis	0.412 ± 0.026	0.497 ± 0.053	$\mathbf{0.862 \pm 0.074^{**}}$	$\mathbf{0.756 \pm 0.122^{**}}$	0.344 ± 0.119	45.5	0.582	0.028	8
	Tis+3	-0.088 ± 0.021	$0.233 \pm 0.031^{\#\#}$	0.526 ± 0.081	0.205 ± 0.090	0.293 ± 0.087	142.9	0.651	0.050	6 (a)
Eukaryotic picophytoplankton	Tis	0.226 ± 0.044	0.266 ± 0.055	0.387 ± 0.042	0.226 ± 0.044	*0.086 ± 0.146*	0.0	0.055	0.577	8
	Tis+3	$\mathbf{0.507 \pm 0.025^{*}}$	$\mathbf{0.644 \pm 0.031^{*\#}}$	$\mathbf{0.638 \pm 0.042^{**}}$	$\mathbf{0.507 \pm 0.025^{**}}$	*0.069 ± 0.143*	0.0	0.038	0.641	8
Cyanobacteria	Tis	0.279 ± 0.009	0.183 ± 0.054	0.596 ± 0.115	0.769 ± 0.185	0.490 ± 0.185	63.7	0.538	0.038	8
	Tis+3	$\mathbf{0.571 \pm 0.021^{*}}$	$\mathbf{0.525 \pm 0.053^{*}}$	$\mathbf{0.846 \pm 0.077^{*}}$	0.913 ± 0.115	0.342 ± 0.113	37.5	0.694	0.039	6 (b)

NGR.DIL1 and NGR.DIL1 + (d^{-1}): phytoplankton net growth rates in undiluted treatments without and with added nutrients, respectively; μ_0 (d^{-1}): phytoplankton potential instantaneous growth rates; μ (d^{-1}): phytoplankton in situ instantaneous growth rates (μ = NGR.DIL1 + g); g (d^{-1}): microzooplankton grazing rates; and %pNPP: percentage of particulate net phytoplankton production daily removed by grazing (% pNPP = $m/\mu \times 100$). Regression coefficient of determination (R^2), number of values (n), and significance level of the regression slope (P-value) are also provided. (a) Non-linear dilution plot (apparent saturated feeding); R^2 was obtained for the linear part of the response); (b) outlier(s) removed for compliance with dilution assumptions. Significant effects of increased temperature on growth and grazing rates are denoted by asterisks, after the highest value (in bold): $^*P < 0.05$; $^{**}P < 0.01$. Significant effects of nutrient addition on net growth rates in undiluted treatments (NGR.DIL1 versus NGR.DIL1 +) are denoted by asterisks after the NGR.DIL1 + value: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$. g values shown in italics are not significantly different from zero (i.e. statistically non-significant slopes)
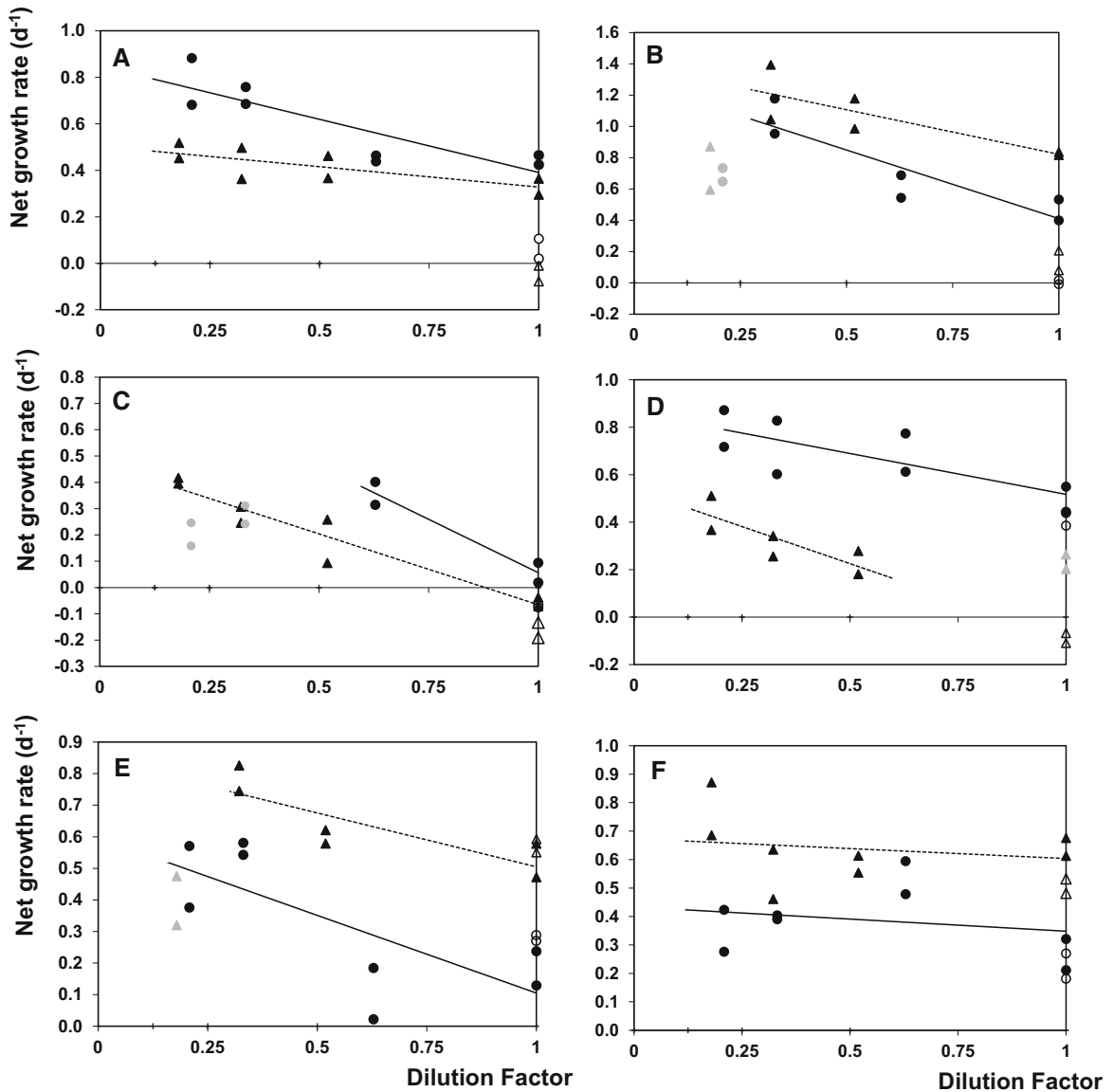
Fig. 5 Relationship between dilution factor and net growth rate of (**A**) natural phytoplankton assemblage, (**B**) planktonic diatoms, (**C**) cryptophytes, **D** other plastidic flagellates, **E** cyanobacteria, and **F** eukaryotic picophytoplankton during dilution experiments undertaken under in situ (circles, T_{is}) and increased (triangles, $T_{is+3°C}$) water temperature (experiment 2). Solid symbols (black and grey) represent net growth rates for dilution treatments amended with nutrients; open symbols represent net growth rates for undiluted samples incubated without nutrients. Regression lines were fitted to net growth rates from treatments with added nutrients (black solid symbols); all regression lines shown have statistically significant negative slopes ($P < 0.05$). Grey bold symbols were not used for linear regressions

($P < 0.01$), μ, and g ($P < 0.05$), in comparison with ambient temperature. However, no significant effects of warming were detected on phytoplankton net growth rates in undiluted sample without added nutrients (NGR.DIL1), an experimental treatment that can be considered a proxy for short-term evolution of

phytoplankton in nature. Warming was also associated with significant declines in μ_0 for both cryptophytes (Fig. 5C) and other plastidic nanoflagellates (Fig. 5D; $P < 0.01$) but showed no significant effects on g and NGR.DIL1. Regarding planktonic diatoms, no significant effects of warming were detected on μ_0, μ, g, or

NGR.DIL1 (Fig. 5B), whereas for smaller-sized phytoplanktonic groups (cyanobacteria and eukaryotic picophytoplankton), warming enhanced NGR.DIL1 and NGR.DIL1+, μ_0, and μ of eukaryotic picophytoplankton (Fig. 5E, F), but showed no significant effects on g (Table 3). Grazing by microzooplankton upon eukaryotic picophytoplankton under both temperatures was not significantly different from 0.

Discussion

Our two short-term microcosm experiments showed that the effects of warming and acidification varied among phytoplankton functional groups, leading to positive, neutral, and negative effects on phytoplankton growth rates. Overall, phytoplankton growth rates decreased with warming, except for smaller-sized groups. Interactions between CO_2 and temperature were negative antagonistic, i.e. the cumulative effects of the two stressors were positive (Piggott et al., 2015), only for the whole assemblage and cryptophytes. Warming was also associated with lower potential and in situ instantaneous growth rates of the phytoplankton assemblage and lower rates of microzooplankton grazing upon phytoplankton.

Diatoms showed a moderate positive response to high CO_2 levels, but no effects of temperature or interactions between these two stressors were observed. Positive effects of high CO_2 on diatom net growth rates, reported for several marine ecosystems (see review by Bach & Taucher, 2019), were previously referred for a winter phytoplankton assemblage from the Ria Formosa coastal lagoon, sampled approximately one month prior to the present study (Domingues et al., 2014). Our study also revealed a significant increase in silica consumption in relation to the control, not only in high CO_2 treatments, but also in the high temperature treatments, without any growth enhancement. This could be attributed to a potential nutrient limitation of the diatom assemblage. Indeed, both the application of nutrient limitation criteria (e.g. N limitation if DIN < 1 M, DIN:P < 10, and Si:DIN > 1: Justíc et al., 1995) and the results from dilution experiments (undiluted samples with and without added nutrients) suggest that nutrients, particularly nitrogen, were a limiting resource to phytoplankton growth. Nitrogen limitation in the Ria Formosa coastal lagoon frequently occurs during summer (Domingues et al., 2015), and was also detected in February 2012 using nutrient enrichment experiments (Domingues et al., 2017a). Nitrate, usually the preferred nitrogen source for diatoms (Domingues et al., 2011b), was equally consumed in all treatments, whereas silica consumption was stimulated in all CO_2 and temperature experimental treatments. This uncoupling between Si consumption and diatom growth under warming conditions suggests luxury consumption of Si by diatoms, that only occurs under non-limiting Si levels and that may result in delayed biomass production (Revilla & Weissing, 2008). Si concentration at the beginning of incubation was indeed non-limiting according to different nutrient limitation criteria (Fisher et al., 1988; Justíc et al., 1995). Nutrient enrichment experiments conducted in February 2012 at the same lagoon location also showed that Si was not limiting phytoplankton growth (Domingues et al., 2017a). Therefore, high CO_2 and warming probably stimulated luxury consumption of Si by diatoms, but nitrogen limitation prevented the occurrence of warming-induced stimulation of net and in situ instantaneous growth. High CO_2 levels probably alleviated the effects of nitrogen limitation, allowing a modest positive increase in net growth rate of diatoms.

Isolated and combined effects of high CO_2 and warming on diatoms vary across the literature, ranging from stimulation, no effects, or inhibition of growth rates (Gao & Campbell, 2014; Hoppe et al., 2018). For instance, high CO_2 levels may enhance growth of a natural diatom assemblage (Domingues et al., 2014). Stimulatory effects of temperature and CO_2, isolated and combined, have been observed for diatom growth (Gao et al., 2012) and for larger-sized diatom species (Wu et al., 2014), although warming may be more influential than CO_2, leading to changes in community structure and reduced species richness (Tatters et al., 2013). These variable responses to short-term perturbations indicate that diatom (and phytoplankton) growth is modulated by a complex matrix of environmental variables that interact synergistically and antagonistically. Differences in experimental setups (e.g. cell density, light and temperature levels) and *taxon*-specific physiology may also lead to differential responses to environmental stressors (Gao & Campbell, 2014).

In contrast to diatoms, net growth rates of cryptophytes decreased under high CO_2 levels, and both net

growth rates (experiment 1) and potential instantaneous growth rates (experiment 2) were also negatively affected under high temperature. However, the combination of these variables induced a negative antagonistic interaction, where the cumulative effects of the two stressors was positive (Piggott et al., 2015), resulting in the alleviation of the negative effects of temperature. Warming was in fact associated with the lowest net growth rates of cryptophytes, in both experiments. This functional group is indeed associated with colder water temperatures (Mendes et al., 2013; Lv et al., 2014) and is dominant off southern Portuguese coast in the autumn and winter (Goela et al., 2014). In the Ria Formosa, however, maximum abundances of cryptophytes have been observed during summer (Pereira et al., 2007) or spring, and minimum abundances during winter, although their range of seasonal variability is reduced (Barbosa, 2006). Studies addressing the effects of high CO_2 and warming on cryptophytes are limited. However, warming was previously reported to have negative effects on cryptophyte growth, when combined with UVR exposure (Halac et al., 2013), even promoting a shift in community composition towards dominance of diatoms and prymnesiophytes, and disappearance of cryptophytes (Vidussi et al., 2011). The reported effects of high CO_2 on cryptophytes are variable across the literature. Decreasing pH levels did not affect growth of several species of cryptophytes up to ~ 7 (Berge et al., 2010), and no changes were observed in alloxanthin concentration (specific marker pigment for cryptophytes) with varying CO_2 levels in microcosms (Biswas et al., 2015). Furthermore, experiments conducted in the Ria Formosa coastal lagoon during winter (February 2012), with manipulation of UVR and CO_2 levels, also showed no significant effects of high CO_2, isolated or associated with UVR exposure, on net growth rates of cryptophytes (Domingues et al., 2014). However, increases in the contribution of cryptophytes to total phytoplankton biomass in high CO_2 microcosms of natural phytoplankton assemblages (Endo et al., 2016) were also reported.

Other growth-regulating variables, such as nutrients and light, may have played a role limiting cryptophytes growth, but nutrient and light enrichment experiments conducted in the Ria Formosa coastal lagoon at the same time with similar water samples showed that neither nutrients nor light intensity were limiting to cryptophytes (Domingues et al., 2017a). The negative responses of cryptophytes to warming and CO_2 could also reflect the beneficial effects of these drivers on grazers, rather than a deleterious direct effect on cryptophyte growth. In addition, microzooplankton grazing pressure on cryptophytes in the Ria Formosa is usually higher than for other phytoplankton groups, particularly diatoms, and the seasonal variability of cryptophytes is also closely associated with grazing by the mixotrophic ciliate *Myrionecta rubra* (Barbosa, 2006). However, the dilution experiments showed no effects of warming on microzooplankton grazing on cryptophytes.

Net growth rates of cyanobacteria were positively affected by temperature in both experiments, but no significant responses to high CO_2 were observed. The latter effect contrast with results derived from an experiment ran two weeks before the present study that reported a reduction in net growth rate of *Synechococcus* in the Ria Formosa coastal lagoon under increased CO_2 levels (Domingues et al., 2014). During summer, growth rate of *Synechococcus* was also reduced under high CO_2 levels; however, nutrient additions (nitrate or ammonium or nitrate + phosphate) counteracted the negative effects of CO_2, leading to increased growth of *Synechococcus* (Domingues et al., 2015). Other studies have reported positive responses of *Synechococcus* to warming and increased CO_2, in terms of cell division and photosynthetic parameters (Fu et al., 2007).

Synechococcus and most cyanobacteria have developed efficient carbon concentrating mechanisms (Rae et al., 2011; Raven & Beardall, 2014), which are downregulated under high CO_2 levels; in theory, more energy would become available for other cellular processes, including cell division (Giordano et al., 2005; Raven et al., 2014). Indeed, non-calcifying phytoplankton will probably benefit from high CO_2, leading to an increase up to 50% in phytoplankton primary productivity (Schippers et al., 2004). However, in some cases, the responses of cyanobacteria, particularly single-celled forms such as *Synechococcus* and *Prochlorococcus*, to high CO_2 were modulated by responses to other growth-regulating variables, such as nutrients, light, and temperature (Lomas et al., 2012).

Warming promoted significant increases in cyanobacteria net growth rates in both experiments, indicating that temperature is a major variable

regulating cyanobacteria growth. Indeed, the effects of temperature on *Synechococcus* growth are much more substantial than the effects of CO_2 (Fu et al., 2007). Concomitantly, ammonium consumption increased significantly under increased temperature, but not in the high CO_2 treatment under in situ temperature, contrasting with nitrate consumption, which responded to neither CO_2 nor temperature increases. Cyanobacteria usually prefer ammonium as their N-source (Dokulil & Teubner, 2000; Domingues et al., 2011b), although they can also take up other N-forms, such as nitrate, nitrite, urea, and even atmospheric nitrogen and amino acids (Flores & Herrero, 2005). The simultaneous increase in ammonium consumption and cyanobacteria net growth rates and their preference for ammonium as N-source suggest that higher temperatures stimulate cyanobacteria nutrient uptake and growth. This was also supported by dilution experiments, where cyanobacteria net growth rates under increased temperature were significantly higher than under ambient temperature.

Net growth rates of eukaryotic picophytoplankton (EPP) in dilution experiments were also stimulated by warming. Like cyanobacteria, EPP usually present maximum abundance during summer in the Ria Formosa coastal lagoon, associated with higher temperature (Barbosa, 2006), and in other temperate lagoons (Pequin et al., 2017). Overall, smaller phytoplankton are potential winners under warming conditions, and this reduction in phytoplankton size can lead to profound alterations in carbon transfer to higher trophic levels and to the deep ocean (Maugendre et al., 2015). Ocean acidification, however, may have a detrimental effect on the growth of smaller-sized cells (Richier et al., 2014).

The response of the phytoplankton assemblage (based on Chl*a*) to high CO_2 and temperature mirrored the individual responses of cryptophytes, with negative effects of temperature and negative antagonist interactions between the two stressors. Although the abundance of all phytoplankton groups decreased during the 2-day incubation, Chl*a* increased in the control and high CO_2 treatment, and declined under high temperature treatments. Cellular chlorophyll content is highly variable, depending on several factors, such as light, nutritional status, and temperature (Behrenfeld et al., 2015). In general, cells subjected to low temperatures usually have higher concentrations of photosynthetic pigments, leading to a more efficient use of light at low temperatures (Valiela, 1995), which can explain the observed decreases in chlorophyll-*a* concentration under high temperature.

Several studies reported positive correlations between temperature and phytoplankton growth in temperate coastal ecosystems (Montagnes & Franklin, 2001; Collos et al., 2005; Barbosa, 2006; Horn et al., 2020). Temperature raises the substrate-saturated reaction rate of RuBisCO, thus increasing potential phytoplankton growth, providing that growth is not limited by other factors (Beardall & Raven, 2004). However, in both experiments, phytoplankton assemblage, along with cryptophytes and other plastidic nanoflagellates, was negatively affected by warming. Indeed, net growth rates, potential instantaneous growth rates, and in situ instantaneous growth rates were all significantly lower under increased temperature in relation to ambient temperature. In addition, and contrary to our working hypothesis, microzooplankton grazing rates were also lower under increased temperature, so warming did not enhance phytoplankton losses due to herbivory as observed in eutrophic waters (Chen et al., 2012). The exception was for herbivory on pico-sized cells, namely eukaryotic picophytoplankton and cyanobacteria. It is also possible that microzooplankton, mainly composed by nanoflagellates, were feeding on heterotrophic prokaryotes. Overall, results suggest that it is likely that this winter phytoplankton assemblage was well acclimated to winter temperatures and, thus, it was negatively affected by higher temperatures. However, had phytoplankton been given more time to acclimate to a higher temperature, their abundance may have increased, as temperature is the main driver of phytoplankton blooms in shallow productive coastal waters (Trombetta et al., 2019). Despite variable phytoplankton growth responses to high CO_2 and temperature and shifts in functional groups, phytoplankton production was not significantly affected by these environmental drivers. The same pattern of changes in growth and community structure and no changes in phytoplankton production were also referred by others (Tortell et al., 2002; Domingues et al., 2014). Reported isolated and combined effects of different drivers on phytoplankton production are indeed contradictory. For instance, warming (Rose et al., 2009) and increased CO_2 (Tortell et al., 2008;

Song et al., 2014) may lead to increased production, but high CO_2 (Feng et al., 2010; Domingues et al., 2014), high temperature, and the combination of both (this study) may also not affect phytoplankton production. In other cases, increases in phytoplankton production due to acidification are only observed at lower temperatures, but this response is constrained by nutrient and light availability (Holding et al., 2015).

Conclusions

Our results showed that short-term variability in different environmental drivers may have varying effects on phytoplankton growth, depending on the functional group. In addition, isolated effects may be counteracted by interactions between those drivers. In general, high temperature and CO_2 did not have the beneficial effects on phytoplankton growth that could be expected, suggesting that the winter phytoplankton assemblage from the Ria Formosa coastal lagoon was well acclimated to ambient conditions and even small, short-term changes in specific drivers may cause significant variability in growth and community structure. Our second hypothesis was rejected, given that warming did not increase microzooplankton grazing on phytoplankton, suggesting that microzooplanktonic organisms were also well acclimated to ambient conditions and were apparently not able to increase their metabolic and feeding rates over a short time scale, following rapid increases in temperature. Alternatively, they may have switched to bacterial prey under warmer conditions. In the context of climate change, long-term experiments that allow the development of adaptive responses, in contrast with the plastic responses observed in short-term studies, are the golden standard to evaluate the impacts of future environmental scenarios. However, many climate-related drivers, such as temperature and CO_2, also vary on shorter time scales and their study will thus provide a basic understanding of ecological and physiological mechanisms that may further impact phytoplankton acclimation and adaptation to future environmental change.

Data availability Upon request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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