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ARTICLE

Impact of increasing water temperature on growth, photosynthetic efficiency, nutrient consumption, and potential toxicity of *Amphidinium* cf. *carterae* and *Coolia monotis* (Dinoflagellata)

Impacto del aumento de temperatura sobre el crecimiento, actividad fotosintética, consumo de nutrientes y toxicidad potencial de *Amphidinium* cf. *carterae* y *Coolia monotis* (Dinoflagellata)

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Resumen.- A nivel mundial, el aumento de la temperatura en ecosistemas marinos podría beneficiar la formación de florecimientos algales nocivos. Sin embargo, la comprensión de la influencia del aumento de la temperatura sobre el crecimiento de poblaciones nocivas de dinoflagelados bentónicos es prácticamente inexistente. Se investigó el impacto del aumento de la temperatura entre 5 y 30°C en dos cepas de dinoflagelados bentónicos aislados del Fleet Lagoon, Dorset, sur de Inglaterra, y su toxicidad potencial fue determinada a través de dos tipos de bioensayos (mortalidad del copépodo *Tigriopus californicus* y actividad hemolítica en eritrocitos de pollo). Las cepas crecieron en monocultivos en medio f/2 (agua de mar enriquecida), suministradas con irradiancias de 35 a 70 µmol m^{-2} s^{-1} bajo un fotoperiodo de 12:12 h (luz/oscuridad). La abundancia, la eficiencia fotoquímica máxima del fotosistema PSII (*Fv/Fm*), el consumo de nutrientes ($N\text{-}NO_3$+$N\text{-}NO_2$ y $P\text{-}PO_4$) y las tasas de crecimiento se determinaron a temperaturas entre 5 y 30°C en monocultivos de *Amphidinium* cf. *carterae* y *Coolia monotis*. El aumento de temperatura causó tasas de crecimiento mayores en las dos cepas examinadas. Temperaturas elevadas (25-30°C) se asociaron con el óptimo crecimiento de *A.* cf. *carterae*, mientras que la máxima tasa de crecimiento en *C. monotis* se registró entre 15 y 25°C. Las tasas de crecimiento de *A.* cf. *carterae* fueron significativamente mayores a las registradas en *C. monotis*. El declive de *Fv/Fm* se asoció con condiciones deficientes de crecimiento para ambas especies. La máxima *Fv/Fm* no se registró asociada a la temperatura de óptimo crecimiento en ambas especies. La mortalidad del copépodo y el efecto lítico en eritrocitos confirmaron la producción potencial de compuestos nocivos en ambas especies de dinoflagelados.

Palabras clave: *Amphidinium carterae*, *Coolia monotis*, tasa de crecimiento, temperatura, toxicidad

Abstract.- The effect of global warming on marine ecosystems may benefit the formation of harmful algal blooms. However, our understanding of the effect of higher water temperatures on the growth of harmful benthic dinoflagellates in the environment is practically unknown. The effect of increasing water temperatures from 5 to 30°C on the growth of two strains of benthic dinoflagellates (*Amphidinium* cf. *carterae* and *Coolia monotis*) isolated from the Fleet Lagoon, Dorset, south of England, was investigated, and their potential toxicity was assessed using two types of bioassays (mortality of the copepod *Tigriopus californicus* and haemolytic activity of chicken red blood cells). Benthic dinoflagellate strains were grown in f/2 medium at irradiances of 35-70 µmol m^{-2} s^{-1} and under a 12h L:12h D photoperiod. Cell abundance, maximum quantum yield of photosystem II (*Fv/Fm*), nutrient consumption ($N\text{-}NO_3$ + $N\text{-}NO_2$ and $P\text{-}PO_4$), and growth rates were determined in dinoflagellate cultures at growth temperatures between 5 and 30°C. Increasing water temperatures caused increased growth rates of the species. Elevated temperatures (25-30°C) were linked to the optimum growth of *A.* cf. *carterae*, while maximal growth rates in *C. monotis* were recorded between 15 and 25°C. *A.* cf. *carterae* had significantly higher growth rates than *C. monotis*. Declining *Fv/Fm* values indicated unsuitable growth conditions in culture for both species. The highest *Fv/Fm* values recorded had no relation to the optimum growth temperature for either species. Copepod mortality and lysis of erythrocytes confirmed that both dinoflagellate species may produce chemical compounds with potentially noxious effects.

Key words: *Amphidinium carterae*, *Coolia monotis*, growth rate, temperature, toxicity

Introduction

More than 30 species of benthic dinoflagellates are toxin-producing microalgae (Hoppenrath *et al*. 2014) and may form harmful algal blooms (HABs) in tropical and temperate aquatic ecosystems worldwide (Nakajima 1981, Penna *et al*. 2005, Shears & Ross 2009, GEOHAB 2012, Hoppenrath *et al*. 2014, Ismael 2014). These microorganisms live epiphytically on macroalgae and seagrasses in coral reef zones, interstitial spaces in marine sediments, and associated with substrata in mangroves (GEOHAB 2012, Hoppenrath *et al*. 2014). In these benthic ecosystems, changes in water temperature determine seasonal trends and densities of microalgae communities (Raven & Geider 1988, Edwards *et al*. 2006). Benthic HAB species have become of global concern because rapid environmental changes in shallow coastal waters (water warming, eutrophication) may increase the development of HAB events worldwide (Peperzak 2003, Beardall & Stojkovic 2006, Edwards *et al*. 2006, Hallegraeff 2010, Bresnan *et al*. 2013). Despite the incipient knowledge of climate change (water warming) on HABs, diverse experimental predictions argue that higher water temperatures in aquatic environment may stimulate the optimum growth of HAB species (Peperzak 2003, Beardall & Stojkovic 2006, Edwards *et al*. 2006, Hallegraeff 2010, Bresnan *et al*. 2013). Nonetheless, there is little understanding of the influence of warmer water temperatures on the growth and toxin production of benthic HAB species in the environment.

The genera *Amphidinium* Clap. et Lachm. *emend.* Flø Jørgensen, S. Murray et Daugbjerg, *Prorocentrum* Ehrenb., *Gambierdiscus* Adachi et Fukuyo, *Fukuyoa* F. Gómez, D. Qui, R.M. Lopez et S. Lin, *Ostreopsis* Schmidt, and *Coolia* Meunier are known to form non-pelagic blooms in both tropical and temperate coastal waters (Morton *et al.* 1992, Gómez *et al*. 2015), and some species are toxic to higher organisms, including humans (Hallegraeff 2003). Noxious events of *Amphidinium* cf. *carterae* Hulburt and *Coolia monotis* Meunier have been documented in coastal waters (Ismael *et al*. 1999, Armi *et al.* 2010, Ismael 2014), but their mechanism of toxicity in the environment remains to be revealed. *A.* cf. *carterae* is recognized as being a fast growing dinoflagellate ($\mu = 2.7$ day^{-1}, Ismael *et al*. 1999) compared to other benthic and planktonic microalgae (Gerath & Chisholm, 1989, Strom & Morello 1998, Lee *et al*. 2003). This species comprised 85% of the total phytoplankton abundance in intertidal pools in the North Arabian Sea (Baig *et al.* 2006). More than 20 secondary metabolites have been identified, isolated, and characterized to molecular level from *A.* cf. *carterae* (Kobayashi & Tsuda 2004). These compounds have demonstrated haemolytic (Nakajima *et al.* 1981, Nayak *et al.* 1997, Meng *et al.* 2010), cytotoxic (Ismael *et al.* 1999, Jeong *et al.* 2003), ichthyotoxic and antifungal activities (Echigoya *et al.* 2005) and are recognized as amphidinolides. To date, amphidinol-3 has been considered the most active secondary metabolite produced by *A.* cf. *carterae*, and it exhibits a potent haemolytic activity against human erythrocytes and an antifungal activity against the ascomycetous fungus *Aspergillus niger* van Tieghem (de Vicente *et al.* 2006).

Toxicological analyses of some strains of *Coolia monotis* have been linked to cytotoxic effects (Nakajima *et al.* 1981) and mortality of both *Artemia* and mice (Rhodes & Thomas 1997, Rhodes *et al.* 2000, 2010). In the North Lake of Tunis, the growth of *C. monotis* cells produced a toxic bloom associated with the mortality of juvenile fish (Armi *et al.* 2010). Few reports have provided evidence of the chemical structure of toxins in *C. monotis*. The first toxin characterized from a strain of *C. monotis* was cooliatoxin (a potent cardiac stimulant, Holmes *et al*. 1995); some other secondary metabolites have also been reported, *e.g.*, cooliatin, an unprecedented dioxocyclononane (Liang *et al.* 2009), and unknown analogues of a polyether compound (Rhodes *et al*. 2000).

The Fleet Lagoon is one of the largest tidal and valued lagoons in the UK (Weber *et al.* 2006). It is a shallow marine ecosystem (depth < 2 m) that possesses a rich diversity of phytoplankton species (Nascimento 2003), including the harmful genera *Amphidinium* and *Coolia*. Harmful effects of these benthic genera in English waters have not yet been reported. In coastal waters of the UK, many uncertainties persist with regard to how water warming might accelerate the reproduction, bloom formation and toxin production by harmful dinoflagellates (Bresnan *et al*. 2013), such as *A.* cf. *carterae* and *C. monotis*. To broaden our understanding of how temperature modulates the dynamics of benthic dinoflagellate growth, this work aimed (1) to determine the effect of increasing sea water temperature on the growth and photosynthetic efficiency (*Fv/Fm*) of *A.* cf. *carterae* and *C. monotis*, and (2) to assess the potential toxicity of the isolates based on mortality of harpacticoid copepods and lysis of red blood cells.

Material and methods

Sampling and establishment of cultures

The epibenthic dinoflagellates *Amphidinium* cf. *carterae* and *Coolia monotis* were isolated from samples of the native macroalgae *Chaetomorpha linum* (O.F. Müll.) Kütz. and *Ulva lactuca* L., and from the seagrasses *Ruppia maritima* L., *Zostera noltii* Hornem. and *Z. marina* L. collected in May 2008 from shallow brackish waters of the Fleet Lagoon

(50°36'40''N and 02°31'10''W). Macrophytes and seagrasses were gently collected underwater by hand and placed into plastic bags with seawater (salinity 28-30). Sampling bags contained 1-3 species of the macroalgae listed above and were maintained in the dark, in a cold room (5°C) for up to 3 days prior to microscopic analysis. Plastic bags were gently shaken for 30 s to detach dinoflagellates from macrophytes. Aliquots of ~0.5 ml (3-4 ml of water in total) were examined under a Nikon Eclipse E200 compound microscope, and single cells of *A.* cf. *carterae* and *C. monotis* were isolated using glass micropipettes. Microalgal monocultures were started in 96-well plates (Thermo Scientific Nunc, USA) filled with f/2 medium (Sigma G0154, Guillard & Ryther 1962) prepared with filtered seawater (0.2 µm) from the Fleet Lagoon (salinity 28-30). Growing cells in wells were later transferred to 250 ml conical flasks filled with f/2 culture medium. Cultures of epibenthic dinoflagellates were initiated at growth temperatures of 18-20°C, with a 12:12 h light:dark photoperiod, under irradiances of 35-70 µmol photons m^{-2} s^{-1}. All monocultures were non-axenic.

Growth experiments

Growth experiments were carried out with single strains of *A.* cf. *carterae* and *C. monotis*. The species were grown in 1L of f/2 media (Guillard & Ryther 1962). The initial cell abundances were on the order of magnitude of 10^3 and 10^2 cells mL^{-1} in *A.* cf. *carterae* and *C. monotis* cultures, respectively. Each species was grown in triplicate cultures at water temperatures of 5, 10, 15, 20, and 25°C; duplicates were used at 30°C. Experiments were conducted in incubators (Mercia Scientific, Southam, Warwickshire, England, UK) under an irradiance range of 35-70 µmol photons m^{-2} s^{-1}. Highest irradiances were measured close to lamps (white light illumination). Dinoflagellate taxa were maintained in exponential growth (18-20°C) for at least 4 weeks, and the acclimation time of taxa was two days at each growth temperature prior to growth experiments. Therefore, this study was constrained to short-term acclimation responses in *A.* cf. *carterae* and *C. monotis* within the range of growth temperatures, although some studies have proved that short-term temperature acclimation of algae did not alter metabolic responses when rapid environmental changes occur (Michel *et al*. 1989, Ritter *et al*. 2014). *A.* cf. *carterae* and *C. monotis* cultures were sampled every 2 and 4 days for a period of 4 and 6 weeks, respectively. Growth experiments were performed at two periods: first, at temperatures between 5 and 15°C and later from 20 to 30°C. Cell abundance and maximum quantum yield of photosystem II (PSII) (*Fv*/*Fm*) charge separation were measured in algal cultures as described below.

Cell abundance estimation

Culture flasks were gently mixed before sampling under a laminar flow fume hood. Culture sub-samples (10 ml) were collected in glass tubes and fixed with glutaraldehyde (1% final concentration). Cell counting was performed using a Coulter Counter Multisizer III with a 70 µm aperture tube. Cell abundances in each sub-sample were quantified in triplicate. To validate the quantification method, 10 sampling points over the growth curves were compared to direct cell counts using a 1-mL Sedgwick-Rafter chamber, and results showed > 95% accuracy. Growth rates were computed from the slope of a linear plot of Ln-transformed cell counts over the exponential growth phase.

Maximum quantum yield of PSII (*Fv*/*Fm*)

The physiological state of cells (*e.g.*, nutrient stress) in non-aerated cultures was determined by measuring the maximum quantum yield of PSII charge separation, which measures the probability that the energy of a photon absorbed will be used for photosynthesis, an indicator of photosynthetic efficiency (*Fv*/*Fm*) (Moore *et al*. 2008, Suggett *et al*. 2009, From *et al*. 2014). Measurements were carried out by blue light flashes at 440 nm with a Fast Repetition Rate Fluorometer (FRRF, Chelsea Technologies Group Ltd, Surrey, UK) enabled for a saturation of PSII of 100 flashlets 1.1 µs at 1.1 µs time intervals. Values of *Fv/Fm* were acquired using the manufacturer's software using the following equations:

$$Fv = F_m - F_o$$

$$Fv/Fm = \frac{(Fm - Fo)}{Fm}$$

where *Fv* is the variable Chl-*a* fluorescence; F_o and F_m are the minimum and maximum, respectively, *in vivo* Chl-*a* fluorescence yield (relative) in a dark-adapted state. The reaction centers of PSII were relaxed in the dark for 25 min before a number of blue light flashes at 440 nm permitted the determination of F_o and F_m using a 4.5 ml quartz cuvette. Six single FRRF measurements were performed per replicate, and *Fv*/*Fm* values were averaged at each growth temperature using the FRRF software FastInP (Chelsea Technologies Group, Ltd, Molesey, Surrey, England, UK). F_v/F_m in the cells was analyzed during the lag, exponential and stationary growth phases based on samples taken for cell counts.

Nutrient consumption ($N\text{-}NO_3$+$N\text{-}NO_2$ and $P\text{-}PO_4$)

Nutrient analyses were conducted every 4-6 days. Thirty ml of culture were collected and filtered through GF/F filters, and the filtrate was kept in plastic bottles at -20°C before nutrient

analysis. Samples were defrosted at room temperature and diluted 1/100 using a solution of NaCl+MilliQ water (40%). $N\text{-}NO_3$+$N\text{-}NO_2$ and $P\text{-}PO_4$ were determined in filtered water samples with a QuAAtro segmented flow autoanalyzer and standard colorimetric techniques (Grasshoff 1976, Kirkwood 1996). Each sample was analyzed in duplicate, and average values were used for each sampling point in replicate cultures. The detection limits of $N\text{-}NO_3$+$N\text{-}NO_2$ and $P\text{-}PO_4$ were 0.03 µmol L^{-1} and 0.01 µmol L^{-1}, respectively. Nutrient analysis was carried out at the Biochemistry Laboratory at the National Oceanography Centre, Southampton, England, UK. Total nutrient ($N\text{-}NO_3$+$N\text{-}NO_2$ and $P\text{-}PO_4$) removal from culture media was calculated as follow:

$$\text{Nutrient consumption } (\mu\text{mols L}^{-1}) = \frac{Co - Ci}{\text{N}}$$

where *Co* is nutrient concentration (µmols L^{-1}) at an early exponential growth phase; *Ci* is the nutrient concentration at the end of the exponential growth; N was the change in cell concentration generated during the time (days) of *Co* and *Ci*. Total nutrient consumption ($N\text{-}NO_3$+$N\text{-}NO_2$ and $P\text{-}PO_4$ µmols $cell^{-1}$) over the exponential growth phase was then converted to $N\text{-}NO_3$+$N\text{-}NO_2$ and $P\text{-}PO_4$ picomols $cell^{-1}$ day^{-1}.

Haemolytic assay

Chicken Erythrocyte Lysis Assay (ELA) was conducted to detect and quantify haemolytic compounds synthesized by *A.* cf. *carterae* and *C. monotis*. This study followed the method applied by Eschbach *et al.* (2001) and Neely & Campbell (2006), although in our study we used chicken Red Blood Cells (RBCs) rather than fish RBCs. Chicken RBCs were provided by Seralab, UK, in 10% Alsevers solution, and they were used in bioassays within two weeks of delivery. Algal extracts were prepared with different cell abundances of *A.* cf. *carterae* (0.01, 0.05, 0.25, 0.5, 1, and 2×10^6 cells mL^{-1}) and *C. monotis* (0.2, 0.5, 1, 2, 3, 4, 5, 10, 20, and 30×10^3 cells mL^{-1}), and the percentage of haemolytic activity was estimated for the range of concentrations. Erythrocytes were incubated with the algal extracts in the dark at 20°C for 18 h prior to quantifying spectrophotometrically the lytic response of haemolytic compounds.

Preparation of algal extracts

Algal extracts were prepared from *A.* cf. *carterae* and *C. monotis* cells at the exponential growth phase only. These species were grown in cultures at 20°C using the growth conditions mentioned above. Cell pellets (10 mL of culture) were made and separated from culture medium by centrifugation (10 min at 3500 rpm). Pellets were rinsed in 2 ml of assay buffer (150 nM NaCL, 3.2 mM KCL, 1.25 mM $MgSO_4$, 3.75 mM $CaCl_2$, and 12.2 mM TRIS base; pH adjusted to 7.4 with HCL) to remove remnants of f/2 medium. To make algal extracts, cell pellets were disintegrated in 2 mL of assay buffer using a pulse sonicator with a 70% duty cycle for 2 min. Algae extracts were maintained in ice during and after sonication.

Erythrocyte lysis assay

Prior to haemolytic bioassays, Alsevers anticoagulant was removed from chicken RBCs. Samples of RBCs (250 µL) were suspended in 50 mL of assay buffer. These suspensions of erythrocytes were centrifuged (10 min at 3500 rpm at 4°C) twice in 50 mL of assay buffer, and the supernatant was discarded. Rinsed RBCs without anticoagulant were added to 50 mL of assay buffer and yielded ~2.50×10^6 cells mL^{-1}. This suspension of erythrocytes was used in bioassays, and 5 mL was used to prepare separately positive and negative controls. Positive controls were prepared by sonication (2 min in a 70% duty cycle) to produce 100% lysis, whereas incubated erythrocytes (18 h) in assay buffer alone were considered negative controls. To determine the effect of potential haemolytic compounds on RBC algal extracts were titrated in bioassay to estimate the percentage of haemolytic activity at different cell concentrations. Assays of RBCs combined with algal extracts were carried out in triplicate (5 mL each) using 15 mL centrifuge tubes. Haemolytic assays were incubated in the dark at 20°C for 18 h. Tubes were centrifuged at 4°C for 10 min (3000 rpm) after incubation, and 3 mL samples were used to determine absorption measurements by spectrophotometry (ATI UNICAM 8625 UV/VIS) at 415 nm. Saponin (Sigma-Aldrich 84510) was used in this study as a chemically defined haemolytic agent (Eschbach *et al.* 2001). The haemolytic activity of algal compounds produced by *A.* cf. *carterae* and *C. monotis* was determined based on a reference curve of RBC lysis induced at different concentrations of saponin (Fig. 1). Saponin concentrations ranged from 1 to 50 µg mL^{-1}, and complete lysis (100%) of chicken RBCs was determined at 6 µg mL^{-1}.

Copepod bioassays

The effect of potential toxicity was determined by the mortality of the harpacticoid copepod *Tigriopus californicus* (Reefphyto Ltd, Bristol, UK) that was cultured in 10 L flasks of filtered seawater (0.22 µm), with salinities of 28-31, for a period of 3-4 weeks. Copepod cultures were grown in the laboratory (at room temperature of 14-20°C) in the shade with a natural photoperiod of daylight. *Tigriopus californicus* was grown *in vitro* for a period of 3-4 weeks. Copepods were fed in a culture of mixed chlorophytes, diatoms and cyanobacteria provided by Reefphyto Ltd, Bristol, England, UK. Young adult copepods

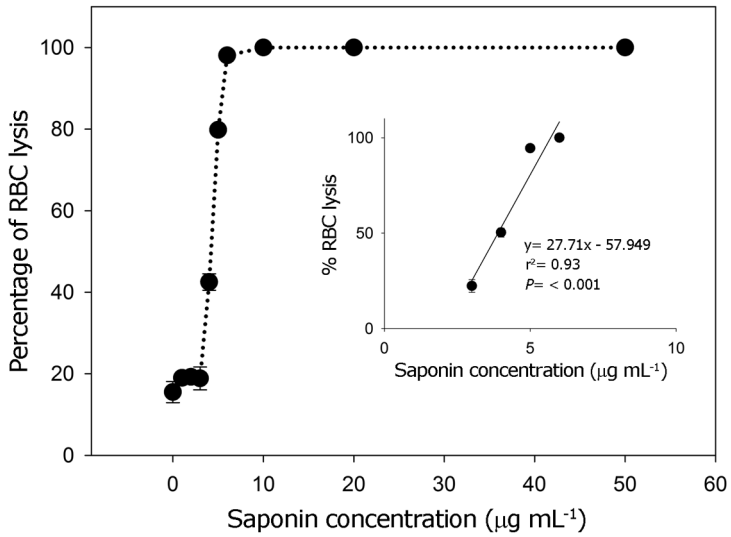

Figure 1. Calibration curve standardized to percentage of RBC lysis at different concentrations of saponin (dotted line and black circles) and linear regression based on haemolytic activity of RBC (inner plot) / Curva de calibración estandarizada a porcentaje de lisis de eritrocitos bajo diferentes concentraciones de saponina (línea punteada y círculos negros) y regresión lineal basada en la actividad hemolítica de las células rojas (gráfica interior)

were used in feeding bioassays. Specimens were isolated from cultures by filtering 2-3 L of water using a net mesh (100 µm). Copepods were briefly rinsed (< 30 s) with distilled water on the mesh to remove debris and food material from their bodies. There was no evidence that copepods were affected by rinsing. Copepods were kept in clean and filtered seawater (0.2 µm) for 5 days without food before testing. Mortality of copepods was tested in 96-well plates (Thermo Scientific Nunc, USA). Bioassays tested 6 abundance treatments of the cultured species were as follows: 2.45, 6.12, 12.2, 30.6, 61.2 and 122.2 ($\times 10^3$) cells mL^{-1} for *A.* cf. *carterae* and 0.11, 0.26, 0.53, 1.31, 2.62, 5.24 ($\times 10^3$) cells mL^{-1} for *C. monotis*. Six replicates of each cell treatment were tested, and average mortality was expressed as a percentage. One replicate contained one starved copepod per well. Mortality assays of *A.* cf. *carterae* and *C. monotis* lasted 9 and 12 days, respectively.

Statistical analyses

Calculated growth rates and *Fv/Fm* values were tested for statistical differences using one-way analysis of variance (ANOVA). The software SigmaPlot version 12.3 was used for all statistical analyses including tests of multiple comparisons in the range of growth temperatures (5-30°C).

Scanning electron microscopy

A volume of 50 mL of *A.* cf. *carterae* (10^5 cells mL^{-1}) and 100 mL of *C. monotis* (10^4 cells mL^{-1}) at late exponential growth phase were concentrated by centrifugation for 15 min at 950 rpm. Supernatant was removed, and cell pellets were fixed in 1.5 ml of 0.1 M PIPES buffer (1,4 Piperazine bis (2-ethanosulfonic acid, pH 7.2) combined with glutaraldehyde (3%) and formaldehyde (4%). Cells were maintained at 4°C for 24 h during fixation. Fixed cells were transferred to silicate coated cover slips by pipetting. Cells were left to settle on the cover slips for 20 min. Cover slips were rinsed twice in 0.1 M PIPES buffer. Samples were dehydrated in series of graded ethanol concentrations (30, 50, 70, 95, and 100%) for 10 min each. Samples were freeze-dried in a critical point dryer, mounted on stubs, and sputter coated for 10-15 min before scanning electron microscope (SEM) observations with a FEI Quanta 2000 SEM at 10-15 kV. Microscopy was carried out at the Biomedical Imagining Unit at the University of Southampton.

Results

The harmful benthic dinoflagellates *Amphidinium* cf. *carterae* and *Coolia monotis* were successfully grown in monocultures at temperatures between 5 and 30°C. Microscopic observations of cultured cells and SEM microphotographs of *A.* cf. *carterae* and *C. monotis* determined distinctive features of the taxa in study (Fig. 2).

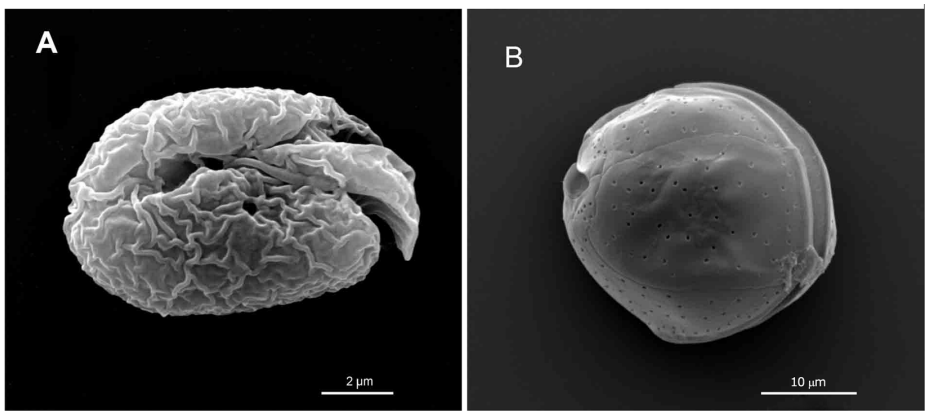

Figure 2. Scanning electron micrographs: *Amphidinium* cf. *carterae* (A) and *Coolia monotis* (B) clonal cells isolated from shallow waters of the Fleet Lagoon, Dorset, United Kingdom / Micrografías electrónicas de barrido de células clonales de *Amphidinium* cf. *carterae* (A) y *Coolia monotis* (B) aisladas de aguas someras del Fleet Lagoon, Dorset, Reino Unido

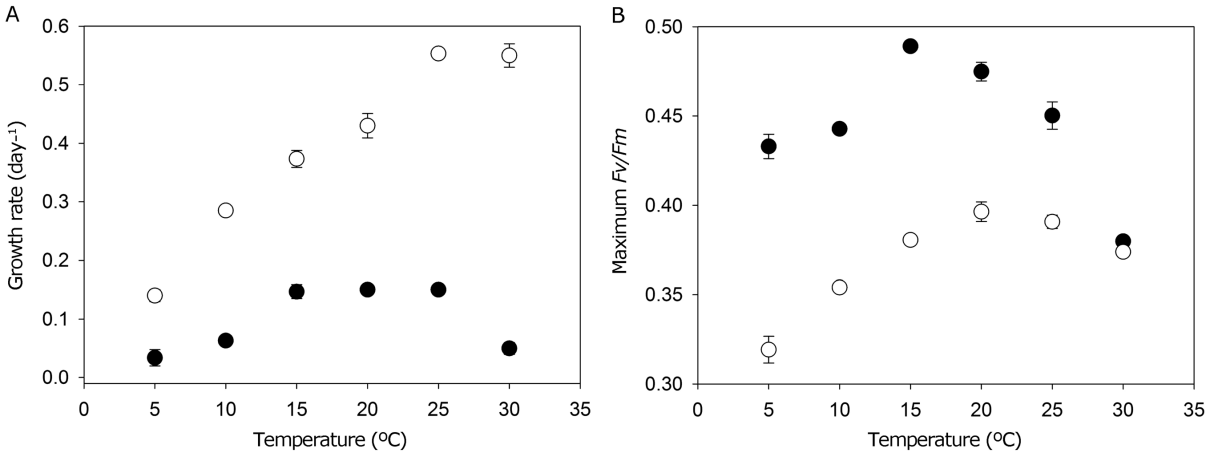

Figure 3. Changes in cell growth rates (A) and maximum photosynthetic efficiency (*Fv/Fm*) (B) in *Amphidinium* cf. *carterae* (white circles) and *Coolia monotis* (black circles) cultures grown at increasing water temperatures between 5 and 30°C / Cambios en las tasas de crecimiento (A) y el máximo de eficiencia fotosintética (*Fv/Fm*) (B) en monocultivos de *Amphidinium* cf. *carterae* (círculos blancos) y *Coolia monotis* (círculos negros) al aumentar la temperatura entre 5 y 30°C

Growth rates and *Fv/Fm*

Optimum growth temperature differed between *A.* cf. *carterae* and *C. monotis*. The former reached its highest growth rate (0.5 day^{-1}) at the most elevated temperatures (25-30°C), while the latter had an optimum growth rate of 0.15 day^{-1} between 15 and 25°C. *A.* cf. *carterae* cultures exhibited higher growth rates (2.5-11 times, 0.1 day^{-1} every 5°C) than those of *C. monotis* as water temperature increased. Therefore, growth rates of *A.* cf. *carterae* proved to be statistically different (P = 0.05) from those in *C. monotis* cultures (Fig. 3). The highest water temperature (30°C) induced significant growth and *Fv/Fm* impairment in *C. monotis* cells (Fig. 3) compared to values at the optimum growth temperatures (15-25°C). Also, declining *Fv/Fm* values throughout the stationary growth phase were indicative of poor photosynthetic performance in cultures of both species. Cultures of *A.* cf. *carterae* changed two orders of magnitude (10^3 to 10^5 cell mL) in cell abundance at temperatures above 10°C, while *C. monotis* cell concentration changed only an order of magnitude in the range of growth temperatures. The elevation of water temperature caused increasing *Fv/Fm* values in *A.* cf. *carterae* and *C. monotis* (Fig. 3). Moreover, the exponential growth phase was characterized by increasing *Fv/Fm* values in both species

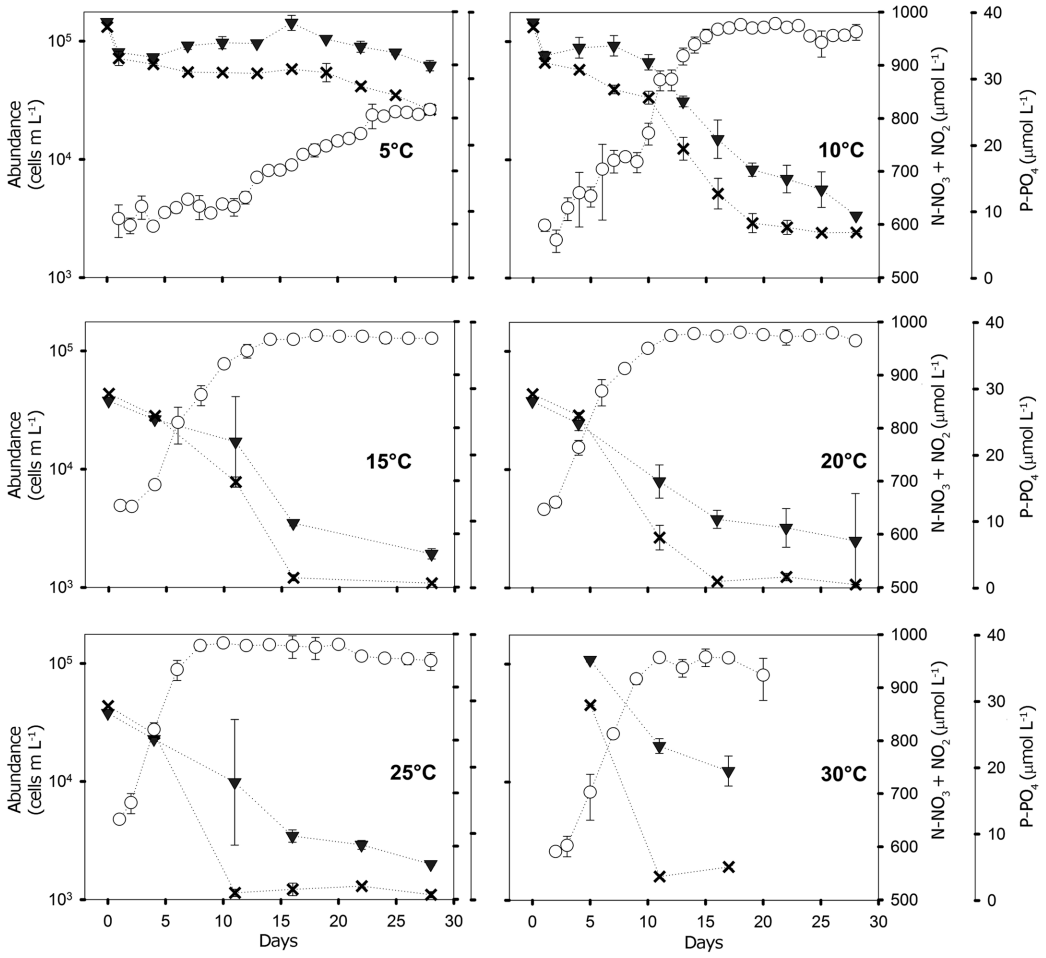

Figure 4. N-NO_3+NO_2 (black triangles) and P-PO_4 (black crosses) consumption (µmol L^{-1}) during the cell growth (white circles) of *Amphidinium* cf. *carterae* in cultures maintained in f/2 medium at growth temperatures between 5 and 30°C / Consumo de N-NO_3+NO_2 (triángulos negros) y P-PO_4 (cruces negras) (µmoles L^{-1}) durante el crecimiento celular (círculos blancos) de *Amphidinium* cf. *carterae* en cultivos mantenidos en medio f/2 a temperaturas de crecimiento entre 5 y 30°C

cultures. Peaks of *Fv/Fm* occurred towards the late exponential growth phase. Highest *Fv/Fm* values mismatched the optimum growth temperature in both species (Fig. 3). However, *Fv/Fm* values in *A.* cf. *carterae* cells did not prove to be statistically different at growth temperatures between 15-30°C, despite differences in growth rates (Fig. 3). Surprisingly, *Fv/Fm* values in *C. monotis* cells were statistically different at all growth temperatures ($P < 0.001$), even within the range of optimum growth temperature (15-25°C) during the exponential growth phase for both species (Figs. 4-5). Despite having lower growth rates, *Fv/Fm* values in *C. monotis* were higher than those in *A.* cf. *carterae*, except at 30°C (Fig. 3).

Nutrients

High consumption of nutrients (N-NO_3+N-NO_2 and P-PO_4) occurred as a result of increasing temperature from 5 to 25°C, particularly during the exponential growth phase of both species (Figs. 4 and 5). The most elevated temperature (30°C) caused the lowest nutrient consumption for *A.* cf. *carterae* despite being linked to the range of optimum growth (25-30°C). The highest N-NO_3+N-NO_2 and P-PO_4 consumption occurred at water temperatures between 20 and 25°C (Figs. 4 and 5). *A.* cf. *carterae* and *C. monotis* had no nitrogen deprivation during growth because N-NO_3+N-NO_2 concentrations were never below 500 µmol L^{-1} within the range of growth temperatures (Figs. 4 and 5). Therefore, the initiation of the stationary growth phase was not a consequence of N-NO_3+N-NO_2 availability.

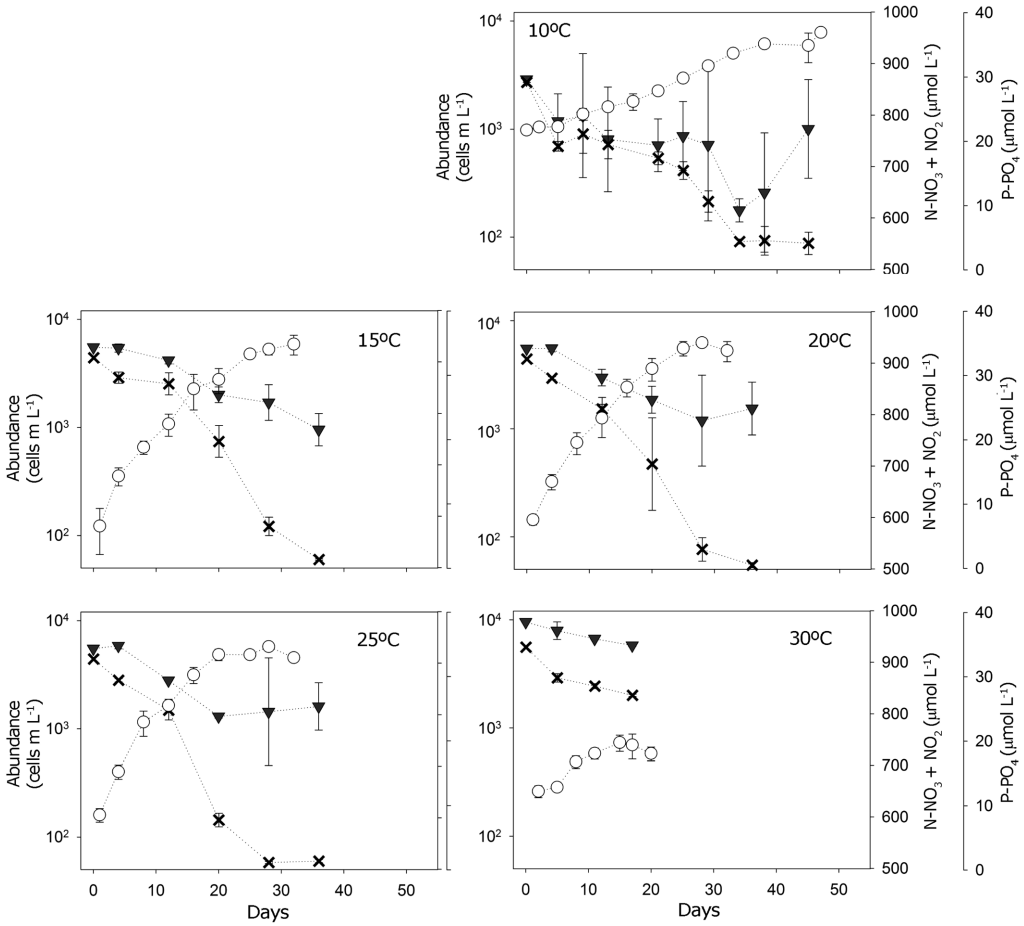

Figure 5. $N\text{-}NO_3+NO_2$ (black triangles) and $P\text{-}PO_4$ (black crosses) removal (µmol L^{-1}) during the cell growth (white circles) of *Coolia monotis* in cultures grown in f/2 medium at growth temperatures between 5 and 30ºC / Consumo de $N\text{-}NO_3+NO_2$ (triángulos negros) y $P\text{-}PO_4$ (cruces negras) (µmoles L^{-1}) durante el crecimiento celular (círculos negros) de *Coolia monotis* en cultivos crecidos en medio f/2 a temperaturas de crecimiento entre 5 y 30°C

$P\text{-}PO_4$ limitation occurred during the stationary growth phase, particularly at culture temperatures between 15 and 25°C (Figs. 4 and 5). Furthermore, $P\text{-}PO_4$ limitation was recorded earlier in *A.* cf. *carterae* (~day 10) than in *C. monotis* cultures (days 28-36) at the optimum cell growth temperatures. During exponential cell growth, $N\text{-}NO_3+N\text{-}NO_2/P\text{-}PO_4$ ratios in both species were lower than the Redfield $N\text{-}NO_3+N\text{-}NO_2/P\text{-}PO_4$ ratio (16:1) and decreased at higher temperatures (Table 1). $N\text{-}NO_3+N\text{-}NO_2$ and $P\text{-}PO_4$ consumption per cell was calculated for both species, but results did not show a pattern of higher removal at increasing water temperatures (Fig. 6). Surprisingly, highest $N\text{-}NO_3+N\text{-}NO_2$ and $P\text{-}PO_4$ consumption per cell was not related to the optimum growth temperature for both dinoflagellate taxa (Fig. 6). Nutrient analyses proved to be highly variable in *C. monotis* cultures at 5°C and are not shown in this study.

Table 1. $N\text{-}NO_3+N\text{-}NO_2/N\text{-}PO_4$ ratio values ± standard deviation (SD) determined during the exponential growth phase of *A.* cf. *carterae* and *C. monotis* at temperatures between 5 and 30°C. Values (±SD) were obtained from duplicate (**) and triplicate (***) cultures / Proporciones de NO_3+NO_2/PO_4 determinadas durante la fase de crecimiento exponencial de *A.* cf. *carterae* y *C. monotis* a temperaturas entre 5 y 30°C; los valores (±SD) se obtuvieron de cultivos en duplicado (**) y triplicado (***)

Temperature (°C)	NO_3+NO_2/PO_4 ratios	
	A. cf. *carterae*	*C. monotis*
5	16,9 (±2,33)**	-
10	10,0 (±0,70)**	9,1 (±0,95)***
15	8,2 (±0,35)***	7,0 (±0,21)***
20	7,0 (±0,78)***	5,3 (±0,24)***
25	4,3 (±3,09)***	5,4 (±0,02)***
30	9,7 (±1,48)**	10,3 (±3,86) **

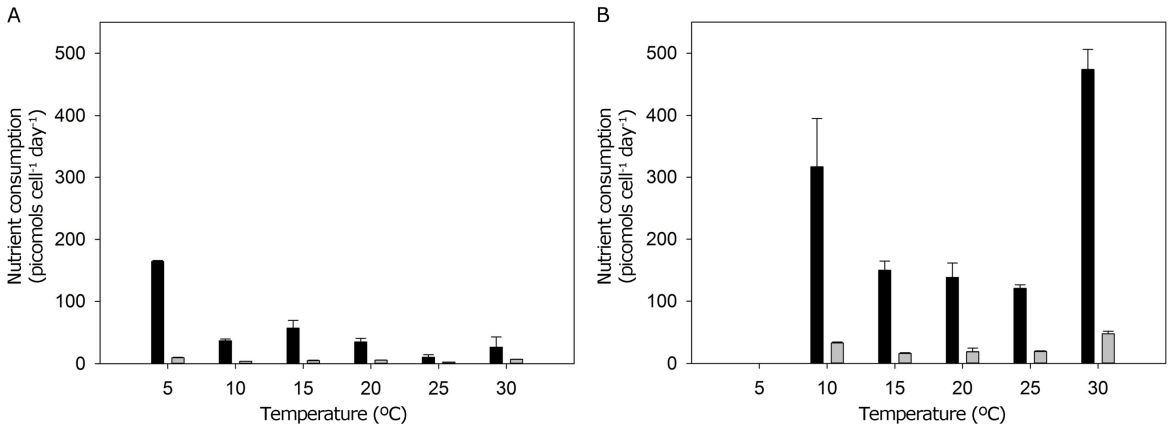

Figure 6. Calculated N-NO_3+NO_2 (black bars) and P-PO_4 (gray bars) cell consumption (picomols cell^{-1} day^{-1}) during the exponential cell growth of *Amphidinium* cf. *carterae* (A) and *Coolia monotis* (B) at water temperatures between 5 and 30°C / Cálculo del consumo celular de N-NO_3+NO_2 (barras negras) y P-PO_4 (barras grises) (picomoles cél^{-1} día^{-1}) durante el crecimiento exponencial de *Amphidinium* cf. *carterae* y *Coolia monotis* a temperaturas entre 5 y 30°C

Copepod mortality

The harpacticoid copepod *Tigriopus californicus* was fed with a wide range of *A.* cf. *carterae* and *C. monoti*s cell abundances for 10 and 12 days, respectively. All the treatments showed high mortality percentages, suggesting harmful effects of both benthic dinoflagellates (Fig. 7). However, the effect of increasing *A.* cf. *carterae* abundance (24.5×10^2-122.5×10^3 cell mL^{-1}) in the feeding assay did not cause either higher or faster mortality of *T. californicus*. High mortality rates (50-100%) from *A.* cf. *carterae* ingestion occurred from day 4 onwards, but, surprisingly, the most elevated cell abundances (61.2 and 122.5×10^3 cell mL^{-1}) only caused 50 and 83% mortality, respectively (Fig. 7). Feeding assays supplemented with *C. monotis* caused higher mortality as cell abundance increased. Even at low abundance, *C. monotis* caused > 50% mortality on day 7 while higher concentrations resulted in 100% mortality as early as on day 5. Control tests of *T. californicus* (fed on non-toxic algae) reached a maximum mortality of 33.3% (day 10) and 16.6% (day 12) at the end of the feeding assays of *A.* cf. *carterae* and *C. monotis*, respectively (Fig. 7).

Haemolytic activity on erythrocytes

The detection and lytic activity of haemolytic compounds from *A.* cf. *carterae* and *C. monotis* were assessed on chicken erythrocytes with crude microalgal extracts at different cell concentrations. Higher haemolytic activity was recorded at increasing cell concentrations for both species after an incubation period of 18 h at a temperature of ~20°C (Fig. 8). Highest haemolytic activity (95-100%) occurred when erythrocytes were exposed to $0.5\text{-}2.0 \times 10^6$ cell mL^{-1} of *A.* cf. *carterae*. Maximum lytic response was 70% with *C. monotis* extracts at the most elevated cell abundances (3.0×10^4 cell mL^{-1}, Fig. 8). Negative controls (erythrocytes without algae extracts) had lower lysis and values recorded after the incubation period (see above) and were subtracted from results of haemolytic assays with algae extracts.

Discussion

The raising of water temperature in combination with higher nutrient loads in aquatic coastal ecosystems is envisaged to potentially increase the occurrence of future HAB events worldwide (Peperzak 2003, Hallegraeff 2010), particularly in shallow coastal environments, *e.g.*, the Fleet Lagoon, Dorset, south of England. Here, two temperate-tropical potentially toxic benthic dinoflagellates, *A.* cf. *carterae* and *C. monotis*, were grown in monocultures at water temperatures between 5 and 30°C. Both species survived and grew along the regime of water temperatures with optimal growth rates linked to both increasing water temperatures and replete nutrient conditions. These benthic dinoflagellates have been found in tropical, subtropical and temperate coastal waters around the world (Morton *et al.* 1992, Ignatiades & Gotsis-Skretas 2010, Gárate-Lizárraga 2012), particularly in coastal marine environments with mesotrophic and eutrophic waters (Aquino-Cruz 2012, Ismael & Halim 2012), although at present tropical findings of *C. monotis* are in doubt (Momigliano *et al.* 2013). Many benthic dinoflagellates are confined to tropical waters, suggesting that temperature may be a primary influence on growth and

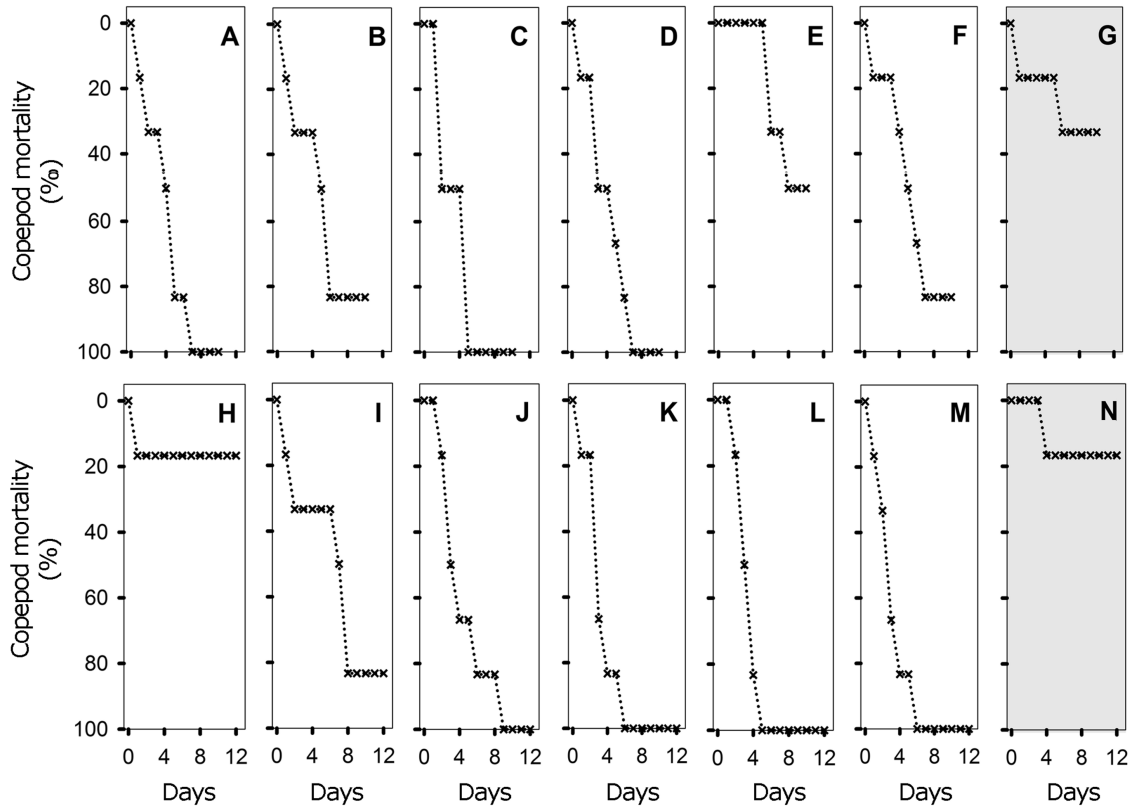

Figure 7. Mortality of the harpacticoid copepod *Tigriopus californicus* fed on increasing microalgal concentrations of *Amphidinium* cf. *carterae* (A: 2.45, B: 6.12, C: 12.2, D: 30.6, E: 61.2 and F: 122.2 (×10³) cells mL⁻¹) and *Coolia monotis* (H: 0.11, I: 0.26, J: 0.53, K: 1.31, L: 2.62, M: 5.24 (×10³) cells mL⁻¹). Right panel (gray color) shows bioassay controls (*T. californicus* fed on non-toxic microalgae) of *A.* cf. *carterae* (G) and *C. monotis* (N). Six replicates were assessed at each cell concentration / Mortalidad del copépodo harpacticoide *Tigriopus californicus* alimentado con concentraciones microalgales en aumento de *Amphidinium* cf. *carterae* (A: 2.45, B: 6.12, C: 12.2, D: 30.6, E: 61.2 y F: 122.2 (×10³) cél mL⁻¹) y *Coolia monotis* (H: 0.11, I: 0.26, J: 0.53, K: 1.31, L: 2.62, M: 5.24 (×10³) cél mL⁻¹). Los paneles de la derecha (color gris) muestran los controles de los bioensayos, *T. californicus* alimentado con microalgas no tóxicas, para *A.* cf. *carterae* (G) y *C. monotis* (N). Seis replicados fueron evaluados a cada concentración celular

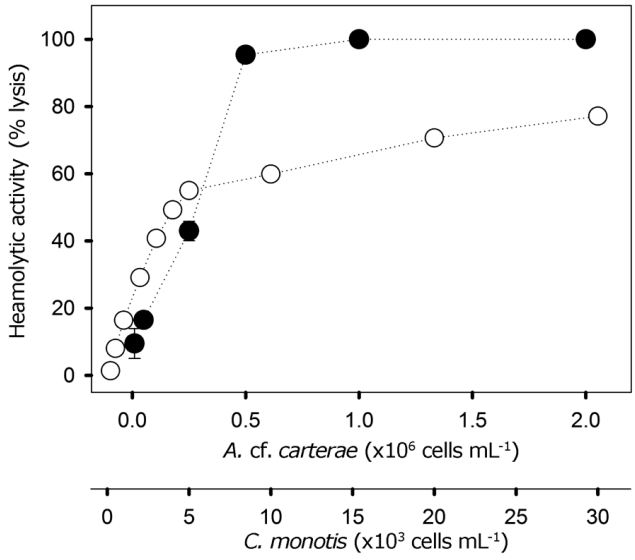

Figure 8. Haemolytic activity of chicken erythrocytes incubated with algal extracts prepared from *Amphidinium* cf. *carterae* (black circles) and *Coolia monotis* (white circles) cells at different abundances / Actividad hemolítica de eritrocitos de pollo incubados con extractos algales de *Amphidinium* cf. *carterae* (círculos negros) y *Coolia monotis* (círculos blancos) preparados con diferentes densidades celulares

distribution (Taylor *et al*. 2003). The Fleet Lagoon is an eutrophic coastal system with several nutrient loadings, *e.g.*, mainly cultivated agriculture (>80%) and livestock (Weber *et al*. 2006), where changes in temperature and nutrient availability may be suitable for harmful events of *A.* cf. *carterae* and *C. monotis*. Of all harmful events caused by dinoflagellates in the Fleet Lagoon, there has been no evidence of an association between higher water temperature and nutrient availability in the development of HABs of *A.* cf. *carterae* and *C. monotis* in this area (Hinder *et al*. 2011, Davidson & Bresnan 2009). HABs of benthic dinoflagellates in the Fleet Lagoon have occurred, but they were commonly ascribed to the genus *Prorocentrum* (Foden *et al*. 2005, Nascimento *et al*. 2005).

The most elevated temperatures (25 and 30°C) in this study caused optimal growth rates of *A.* cf. *carterae* and *C. monotis*. The Fleet Lagoon has been found with surface water temperatures ~25°C during summer (Nascimento 2003) that may provide suitable growth conditions for HAB species. *Amphidinium* cf. *carterae* had high tolerance to elevated temperatures (25-30°C), suggesting an ecological advantage over other harmful benthic dinoflagellates, such as *C. monotis* and *Prorocentrum lima* (Nascimento 2003), in coping with warm conditions in the Fleet Lagoon. Here, warm conditions enhanced the growth rate of *A.* cf. *carterae*, but some strains of the genus *Amphidinium* Clap. et Lachm. have shown decreasing growth rates at temperatures of 31-35°C (Morton 1992). Lee *et al*. (2003) also reported that sustained temperatures above 34°C resulted in death (lysed cells) of *A.* cf. *carterae*. In tropical waters of the Veracruz reef zone, *A.* cf. *carterae* has been shown to be present throughout the year at water temperatures ranging from 24 to 32°C, with highest abundance peaking at the water temperature of 32°C in late spring (Okolodkov *et al*. 2007). In the southern Gulf of California, Gárate-Lizárraga (2012) reported a pelagic bloom caused by *A.* cf. *carterae* at 20°C. Here, *A.* cf. *carterae* not only could grow at elevated temperatures, but also could sustain continuous cell growth at 5°C. This shows a remarkable physiological adaptability of the species for colonization and survival in diverse habitats from warmer tropical to temperate or even colder coastal waters. Generally, physical conditions in microalgal cultures vary, and therefore comparison of growth dynamics between strains can be complicated (Lakeman *et al.* 2009). Table 2 compares culture conditions and growth rates of *A.* cf. *carterae* reported in the literature. Even though some microalgae exhibit a rapid adaptability to high temperatures in short-term experiments, the physiological plasticity and genetic response of many microalgae under future environmental conditions is unknown (Hallegraeff 2015). Also, complex physical interactions (CO_2 dissolution, light, nutrient diffusion, stratification, atmospheric precipitation, and salinity) in the environment at higher temperatures have been insufficiently studied regarding the growth of many harmful microalgae (Hallegraeff 2010). Therefore, future studies should investigate physiological responses (adaptation) of microalgae in the environment, ideally taking into account long-term observations and experiments, to provide a better assessment of the impact of climate change on the growth of harmful dinoflagellates.

Increasing temperatures (5-15°C) triggered higher growth rates of *C. monotis*, but no difference of growth occurred between 15 and 25°C. These ranges of temperatures are common in waters of the Fleet Lagoon (Nascimento 2003) and could sustain blooms of *C. monotis* during favorable growth conditions. Growth experiments lasted a few weeks, and strains had a short period of acclimation between temperatures; therefore, we have not determined whether *C. monotis* might exhibit higher growth rates and adaptability at elevated temperatures in the future. In tropical waters of the Veracruz reef zone of the Gulf of Mexico, *C. monotis* was found throughout the year, with the highest abundance (3.0×10^3 cells g^{-1} seagrass *Thalassia testudinium* Banks ex Konig wet weight) in July at a water temperature of ~28°C and a salinity of 29 (Okolodkov *et al*. 2007). Moreover, Armi *et al*. (2010) reported a bloom of *C. monotis* that occurred at temperatures higher than 22°C between May and July in Tunisian waters that contributed to almost half of the total phytoplankton abundance. Growth rates of *C. monotis* strains under different growth conditions are compared in Table 3.

Fv/Fm (maximum quantum yield of PSII) is a valuable indicator of the physiological state of the cells when either nutrient stress or photoinhibition are experienced by phytoplankton (Moore *et al*. 2008, Suggett *et al*. 2009, From *et al*. 2014). Despite its lack of specificity, *Fv/Fm* could adequately assess nutrient stress in phytoplankton when photoinhibition is not of concern (From *et al*. 2014). Knowledge of *Fv/Fm* values of harmful benthic dinoflagellates is scarce, and comparing *Fv/Fm* data between strains may be difficult due to differences in culture conditions. Ecophysiological studies on photosynthesis-irradiance relationships of one *A.* cf. *carterae* strain have shown a remarkable photoadaptability (light gradient 24-800 µE m^{-2} s^{-1}) of this dinoflagellate, suggesting a biological strategy for prospering in shallow coastal waters (Gerath & Chisholm 1989). This suggests a wide environmental window of the genus *Amphidinium* because a range of growth conditions (*e.g.*, irradiances) have been linked to the optimum growth rate of different *Amphidinium* strains (see Table 2). *Coolia monotis* had higher *Fv/Fm* values than *A.* cf. *carterae*, but growth rates of the former were significantly lower (see Fig. 3). Light history

Table 2. Growth rates and conditions applied during the growth of different strains of *A.* cf. *carterae*; nd= no data available / Tasas de crecimiento y condiciones establecidas durante el crecimiento de diferentes cepas de *A.* cf. *carterae*; nd= datos no disponibles

Species	Growth rate (day^{-1})	Temperature (°C)	Light ($\mu mol\ m^{-2}\ s^{-1}$)	Salinity	Max. cell density ($cells\ ml^{-1}$)	Culture medium	Location of isolation	Reference
Amphidinium carterae	nd	25	56-112	nd	1.9×10^5	SW plus Provasoli's ES supplement	Okinawa, Japan	Nakajima *et al.* 1981
A. carterae	0.39-0.44	15	70 [1]	30	nd	f/10	Tjøme, outer Oslofjord	Sakshaug *et al.* 1983
A. carterae	(27 h generation time)	21	10-70	nd	nd	f/2	nd	Olson & Chisholm 1986
A. carterae	1	20	150-260	nd	2×10^5	f/2	nd	Gerath & Chisholm 1989
Amphidinium klebsii	0.45	27	207 (10% sunlight)	33	nd	K	Knight Key, Florida	Morton *et al.* 1992
A. carterae	nd	nd	28	nd	5.17×10^5	Erdschreiber's soil extract	Mangalore, India	Nayak *et al.* 1997
A. carterae	0.47	nd	nd	30	nd	f/2	nd	Strom & Morello 1998
A. carterae	2.7	25	41-48	nd	nd	GPM (2.5X)	Alexandria, Egypt	Ismael *et al.* 1999
A. carterae	0.5	20	100	nd	nd	f/2	nd	Jeong *et al.* 2003
Amphidinium sp.	1	13.6-32.9	100	38	5×10^5	ASP 7	nd	Lee *et al.* 2003
A. carterae	0.4	16	200	nd	$4\text{-}5\times10^5$	L1 with boric acid, TRIS buffer, and Na_2CO_3	CCAP, UK	Franklin & Berges 2004
A. carterae	0.13-0.55	5-30	35-70	28-33	1.5×10^5	f/2	Fleet Lagoon, UK	This study

Table 3. Growth rates of *C. monotis* determined under different experimental conditions; nd= no data available / Tasas de crecimiento de *C. monotis* determinadas bajo diferentes condiciones experimentales; nd= datos no disponibles

Species	Growth rate (day^{-1})	Temperature (°C)	Light ($\mu mol\ m^{-2}\ s^{-1}$)	Salinity	Max. cell density ($cells\ ml^{-1}$)	Culture medium	Location of isolation	Reference
Coolia monotis	0.15	33	243	33	nd	K (omitting Tris, copper and silica)	nd	Morton *et al*. 1992
C. monotis	0.20	31	243	33	nd	K	nd	Morton *et al*. 1992
C. monotis	0.30	29	243	33	nd	K	nd	Morton *et al*. 1992
C. monotis	0.14	27	243	33	nd	K	nd	Morton *et al*. 1992
C. monotis	0.10	25	243	33	nd	K	nd	Morton *et al*. 1992
C. monotis	0.08	23	243	33	nd	K	nd	Morton *et al*. 1992
C. monotis	0.3	25	100	35	nd	GP	Northland, New Zealand	Rhodes *et al*. 2000
C. monotis	(10 days doubling time)	15	100	27	nd	GP	Northland, New Zealand	Rhodes *et al*. 2000
C. monotis	(3-4 days doubling time)	23	30-90	36	2.5×10^{-3}	Erdschreiber's	Twin Cays, Belize	Faust 1992
C. monotis	0.01-0.32	5-30	35-70	28-33	10.0×10^{-3}	f/2	Fleet Lagoon, UK	This study

signal (*e.g.*, light saturation) or a lack of available reaction centers in small-celled dinoflagellate species may explain lower *Fv*/*Fm* values encountered in *A.* cf. *carterae* (From *et al*. 2014). However, this study was limited to comprehension of the photochemistry (*e.g.*, photoinhibition) in the taxa studied. Higher growth rates of *A.* cf. *carterae* may be explained by rapid metabolic processes of the species. Given that *A.* cf. *carterae* can store nutrients for several generations (Lee *et al*. 2003), cell division may occur in the same manner even though cells have deficiencies in *Fv*/*Fm*.

Higher NO_3+NO_2/PO_4 ratios (24-27:1) were used in growth experiments, and as a result, PO_4 limitation occurred and deterred cell division of the species, particularly at increasing temperatures (15-25°C). Since nitrogen loads in the Fleet Lagoon have historically been higher than those of PO_4, algal bloom formation (Weber *et al.* 2006), duration and permanency could be partially controlled by PO_4 availability. In this study, PO_4 limitation was thought to be the cause of lower *Fv/Fm* values, but biological interpretation must be made cautiously since *Fv/Fm* is not nutrient specific (Kromkamp & Peene 1999).

Amphidinium cf. *carterae* (Ismael *et al.* 1999, Kobayashi & Tsuda 2004, Ignatiades & Gotsis-Skretas 2010, Mandal *et al*. 2011) and *C. monotis* (Nakajima *et al.* 1981, Holmes *et al*. 1995, Rhodes & Thomas 1997, Sugg & Van Dolah 1999) have been recognized as toxin-producing species. Daranas *et al*. (2001) and Kobayashi & Tsuda (2004) have shown the chemical structure of about 20 potential toxins (amphidinolides) linked to *A.* cf. *carterae*. Moreover, antifungal and haemolytic compounds have been reported by Echigoya *et al*. (2005). The first toxin characterized from *C. monotis* was named cooliatoxin, but some studies have shown that not all strains are toxic (Penna *et al*. 2005, Laza-Martínez *et al*. 2011). In this study, bioassays on mortality of the copepod *Tigriopus californicus* and lysis of RBCs suggested the potential toxicity of the strains examined. However, results were limited in relating mortality rates to cell abundance, particularly in the *A.* cf. *carterae* bioassays. Similarly, Baig *et al.* (2006), using wild (2.50×10^2, 10^3, 10^5 $cells\ ml^{-1}$) and cultured (7.20×10^2, 10^3, 10^4 $cells\ ml^{-1}$) *A.* cf. *carterae*, failed to demonstrate a significant mortality effect on the brine shrimp *Artemia salina* (L.) in

feeding experiments. Copepods possess highly sensitive and specific chemoreceptive and selective abilities that influence their grazing behavior (Teegarden & Cembella 1996), and some may exhibit a substantial tolerance to ingestion of toxic algae (Senft *et al.* 2011). Discrepancies found in mortality rates may have been related to the combination of the toxic burden ingested and the potential toxin resistance of harpacticoid copepods. Teegarden & Cembella (1996) commented that some toxins could undergo metabolic transformations in the guts of grazers whereby toxins become less harmful after ingestion.

In conclusion, harmful benthic dinoflagellates from shallow coastal waters may encounter suitable growth conditions as a result of water warming and higher nutrient availability. Global environmental changes and the dynamics of benthic microalgal populations should be assessed with long-term studies in vulnerable coastal areas. More information is needed regarding the mechanisms of toxicity of *A.* cf. *carterae* and *C. monotis* strains at higher trophic levels. In addition, knowledge of chemical transformations of biotoxins from benthic microalgae would be valuable.

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