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CO_2 and phosphate availability control the toxicity of the harmful bloom dinoflagellate *Karlodinium veneficum*

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ABSTRACT: We demonstrated that the toxicity of the harmful bloom dinoflagellate *Karlodinium veneficum* is regulated by both CO_2 concentrations and phosphate availability. Semi-continuous cultures were grown in a factorial experiment under all combinations of 3 CO_2 levels (230, 430 and 745 ppm) and 2 phosphate conditions (0.5 and 20 µM). After steady-state acclimation was achieved, karlotoxin cellular quotas and growth rates were determined in all 6 treatments. This strain produced both types of karlotoxin, KmTx-1 and KmTx-2. Chlorophyll *a*-normalized production of the 2 types of karlotoxins was much higher in P-limited cultures compared with P-replete ones under the same CO_2 conditions. Increasing CO_2 strongly stimulated production of KmTx-1 and decreased production of KmTx-2 in both treatments, but especially in P-limited cultures. Because the KmTx-1 toxin is an order of magnitude more potent than KmTx-2, total cellular toxicity was increased dramatically at high pCO_2, particularly in P-limited cultures. Specific growth rates were accelerated by enriched CO_2 in P-replete cultures, but not in P-limited treatments. Growth rates or toxicity of *K. veneficum* could increase substantially in the future with high CO_2 levels in the ocean, depending on P availability, and so interactions between rising CO_2 and eutrophication could cause major shifts in present day patterns of harmful algal toxin production. These results suggest that over the coming decades, rising CO_2 could substantially increase karlotoxin damage to food webs in the often P-limited estuaries where *Karlodinium* blooms occur.

KEY WORDS: Harmful algae · *Karlodinium veneficum* · Karlotoxin · CO_2 · Phosphate · Dinoflagellates · Ocean acidification · Algal toxin

INTRODUCTION

The frequency and intensity of harmful algal blooms have increased dramatically in recent years, coinciding with a global surge in population density in the coastal zone (Hallegraeff 1993). Agricultural and wastewater enrichment of coastal waters with nutrients is one cause, but is probably not the only one. Today, it is widely recognized that humans are also causing an unprecedented perturbation of the global carbon cycle through burning of fossil fuels and consequent 'greenhouse' warming. In fact, current levels of atmospheric carbon dioxide (CO_2) are predicted to more than double by 2100 (IPCC 2007).

Elevated CO_2 levels in the coming decades could have large consequences for the physiology, nutrient cycling and ecological interactions of marine phytoplankton, including harmful algae. Both experiments with natural phytoplankton communities and laboratory cultures suggest that elevated CO_2 may increase phytoplankton growth (Hein & Sand-Jensen 1997, Burkhardt et al. 1999, Hutchins et al. 2007), or that CO_2 concentrations could affect competition among major marine phytoplankton groups (Tortell et al. 2002, Fu et al. 2007, 2008, Hare et al. 2007, Feng et al. 2009). How major toxic harmful algal groups such as dinoflagellates and raphidophytes will react to globally increasing CO_2 concentrations is, however, just now begin-

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ning to be examined (Rost et al. 2006, Ratti et al. 2007, Fu et al. 2008).

At lower pre-industrial era CO_2 levels or even at present day atmospheric CO_2 concentrations, obtaining enough dissolved CO_2 from the water to support growth can be a problem for some algae. Consequently, many algal groups have evolved carbon-concentrating mechanisms (CCMs) to elevate the concentrations of CO_2 near the active site of CO_2 fixation in the cell, the enzyme Rubisco. Several studies have documented effective CCM activity in some harmful bloom dinoflagellates including *Prorocentrum micans* and *P. minimum* (Nimer et al. 1997, Rost et al. 2006). Internal carbonic anhydrase (CA) activity has been observed in most dinoflagellates studied (Ratti et al. 2007). However, some toxic dinoflagellates appear to have very limited CCM capabilities (Dason et al. 2004), thus raising the possibility of CO_2 limitation in these species. Dinoflagellates may also be directly growth-limited by high pH values (Hansen et al. 2007). Many dinoflagellates have a form II Rubisco that has a particularly low affinity for carboxylation, and is therefore extremely inefficient at fixing CO_2 compared with the form I Rubisco in all other algae (Whitney & Yellowlees 1995, Jenks & Gibbs 2000, Nassoury et al. 2001). Other harmful algal groups such as the raphidophyte *Heterosigma* apparently lack CCMs that would allow them to take up HCO_3^- either directly or indirectly, and hence probably depend only on CO_2 uptake (Nimer et al. 1997). Consequently, it appears that rising CO_2 could favor these particular toxic algal groups over some co-occurring harmless species (Fu et al. 2008).

However, rising CO_2 levels is not the only global change factor that will affect harmful algal blooms. Further increases in coastal eutrophication seem inevitable in parallel with ongoing human population growth and the expansion of industrial agricultural practices. These future increases in nutrient availability are also likely to result in shifts in toxic algal community composition and cell physiology. Many harmful algal species may encounter ideal conditions for bloom formation in a future marine environment enriched in both CO_2 and nutrients.

One widespread harmful algal genus in estuaries such as those along the east coast of the USA and in parts of New Zealand is *Karlodinium.* The species *K. veneficum* is an ichthyotoxic dinoflagellate that is sometimes dominant in estuaries, brackish ponds and coastal aquatic ecosystems of the US Atlantic coast (Adolf et al. 2008, Place et al. 2008). This species often forms blooms that have been associated with massive fish kills, which are believed to be due to the karlotoxin produced by this alga (Deeds et al. 2002, Hall et al. 2008).

It is well known that the synthesis and composition of algal toxins can be affected by environmental conditions (Plumley 1997). Laboratory studies have shown that the production of toxins by *Karlodinium* is related to temperature, salinity and the availability of nitrogen and phosphate. For instance, enhanced karlotoxin production by *Karlodinium* is observed in P-limited cultures (Adolf et al. 2009). This connection between increased cell toxicity and nutrient limitation has also been observed in the production of other algal toxins, such as saxitoxin and domoic acid (Fehling et al. 2004, Frangópulos et al. 2004). As suggested by Guisande et al. (2002), enhanced cell toxicity under nutrient stress may serve to inhibit the growth of other algae, which are competitors for limiting nutrients. In addition, for a mixotrophic algal species, like *K. veneficum*, enhanced cell toxicity may influence its ability to capture prey, and thus help to alleviate nutrient stress (Adolf et al. 2006).

Therefore, previous work suggests that nutrient availability and many other environmental factors can affect the production of toxins by dinoflagellates. This raises the question: Can toxin production also be influenced by changing pCO_2 levels? Documented differences in CCM capabilities between dinoflagellate groups suggest that their growth, and perhaps their toxicity as well, could be affected by the availability of CO_2.

We present here an initial examination of the potential interactive effects of future increases in carbon dioxide (CO_2) and changes in nutrient availability (P) on the growth rates and toxin production of *Karlodinium veneficum*. The aim of this study is to understand how damaging blooms of this harmful bloom species may respond to likely future conditions of elevated CO_2 and P availability.

MATERIALS AND METHODS

Cultures and growth conditions. Experiments used *Karlodinium veneficum* (Center for the Culture of Marine Phytoplankton strain 2936) isolated from the Delaware Inland Bays, USA. Stock cultures were grown at 24°C in 0.2 µm filtered, microwave-sterilized open ocean seawater diluted 25% with MilliQ water to simulate estuarine salinities and enriched with phosphate, nitrogen, vitamin and trace nutrients at the concentrations used in the algal culture medium AQUIL (Morel et al. 1979). Light was provided on a 12 h dark:12 h light cycle using cool white fluorescent bulbs at 120 µmol photons m^{-2} s^{-1}.

Experimental design. Semi-continuous culturing methods were used to measure the effects of PO_4^{3-} availability and/or pCO_2 levels during acclimated

steady-state growth (Hutchins et al. 2007, Fu et al. 2008). Semi-continuous cultures were diluted every 2 or 3 d based on growth rates calculated from *in vivo* fluorescence and cell counts, using medium that was previously adjusted to the appropriate phosphate concentration and pCO_2. The use of *in vivo* fluorescence as a real time biomass indicator to determine daily dilution volumes was validated by subsequent preserved cell counts. Each bottle was diluted individually based on the growth rate calculated for that bottle back to the original biomass immediately after the previous dilution; thus, growth rates were allowed to reach a different steady-state level under each experimental treatment. Cultures were sampled when they were considered fully acclimated to the experimental conditions after statistically invariant growth rates were recorded over 6 to 7 (P-limited) to 10 to 15 (P-replete) generations.

Added HPO_4^{2-} concentrations were 20 and 0.5 µmol l^{-1} for the P-replete and P-limited cultures, respectively, and 320 µM NO_3^- was added to all cultures. The dissolved organic P concentration in seawater is typically ~0.1 µmol l^{-1}, and much of this is refractory and not available to support growth (Hutchins & Fu 2008). Since the concentrations of dissolved inorganic P (DIP) in the medium were 5 to 200 times higher than this, the cultures necessarily had to rely on DIP to support their observed growth. Within each P condition, triplicate bottles were equilibrated by gently bubbling the medium (<1 ml min^{-1}) with 3 different commercially prepared air/CO_2 mixtures (Praxair Gas). Preliminary experiments were carried out to determine whether bubbling affected the growth of the cultures. Growth rates of controls with no bubbling were not significantly different from those of gently bubbled cultures ($p > 0.05$, data not shown).

Carbonate buffer system measurements and pCO_2 treatments. The 3 experimental CO_2 concentrations were chosen to simulate a range of pCO_2 spanning preindustrial atmospheric levels, current concentrations, and the values predicted to occur near the end of this century (IPCC 2007). Seawater medium pCO_2 in the experimental bottles was monitored throughout the experiment by calculation using measurements of pH and total dissolved inorganic carbon (DIC) according to Weiss (1974) and Mehrbach et al. (1973). To ensure that CO_2 levels remained constant during growth, the pH in each bottle was monitored daily using a microprocessor pH meter, calibrated with pH 7 and 10 buffer solutions. For the analysis of total DIC, samples were kept in 25 ml borosilicate flasks free of air bubbles and preserved with 200 µl of 5% $HgCl_2$ l^{-1}, and stored at 4°C until analysis in triplicate. Details of the DIC analyses are provided in Fu et al. (2007). The 3 steady-state experimental CO_2 concentrations used were 232 ± 4.4 (mean ± SD), 431 ± 21 and 744 ± 35 ppm (Table 1); for convenience, these treatments are referred to throughout this paper using rounded-off values of 230, 430 and 745 ppm.

Table 1. Characteristics of the seawater carbonate buffer system in this study. Steady-state seawater medium pCO_2 levels were calculated from measurements (taken every 2nd day) of pH and dissolved inorganic carbon (DIC). Equilibrium constants of Mehrbach et al. (1973) and Weiss (1974) were used. Values include ±SD of the measurements from all 6 experimental bottles at each pCO_2

pCO_2 treatment (ppm)	pH measured	DIC (µmol kg^{-1}) measured	Seawater pCO_2 (ppm) calculated
230	8.37 ± 0.01	1919 ± 33	232 ± 4.4
430	8.16 ± 0.03	2079 ± 17	431 ± 21
745	7.94 ± 0.02	2149 ± 27	744 ± 35

Determination of growth rates and chlorophyll content. Experiments used identical semi-continuous culturing methods to measure phosphate and CO_2 effects during acclimated steady-state growth (Hutchins et al. 2007, Fu et al. 2008). The cultures were diluted with fresh medium every other day by exactly enough volume to reproduce the biomass recorded immediately following the previous dilution, so the cultures were allowed to 'choose' their own growth rate under the experimental conditions. The shortest doubling time for our fastest growing treatment was 2.5 d (see 'Results'), so the cultures were always diluted with fresh medium even before they reached a single doubling, thereby preventing excessive nutrient drawdown or high biomass accumulation and maintaining the cells in constant exponential growth phase. Cell counts and *in vivo* fluorescence (measured with a Turner 10AU fluorometer) were used to monitor growth rates, which were calculated as in Fu et al. (2008). Chlorophyll a (chl *a*) content was measured using a Turner 10-AU fluorometer using the non-acidification protocol (Welschmeyer 1994) as described in Fu et al. (2008).

Liquid chromatography–mass spectrometry quantification of karlotoxin. Karlotoxin concentrations were measured by liquid chromatography–mass spectrometry (LC-MS) using the fact that karlotoxin binds to Teflon (PTFE) quantitatively (Bachvaroff et al. 2008). To estimate chl *a*-normalized toxin content of *Karlodinium*, 5 to 10 ml aliquots from each treatment were filtered onto 0.2 µm polytetrafluoroethylene (PTFE) membrane filters and kept at 5°C until analysis. Analysis of the karlotoxin by LC-MS was performed following the method of Bachvarroff et al. (2008). Before the analysis, the loaded filter syringe was washed with 1 ml of methanol into 2 ml of HPLC water. The samples were injected onto a C8 (LiChrosphere 125 × 4 mm,

5 µm bead size, RP-8, Waters) column and subjected to a 1 ml min^{-1} 10 to 95% methanol:water gradient over 25 min using an HP/Agilent 1100 HPLC. Toxin peaks were detected with optical density (OD) at 225 or 235 nm as appropriate for KmTx-1 or KmTx-2 toxins, respectively. A portion of the mobile phase (⅓ to ⅙) was then passed to the electrospray nozzle of the mass spectrometer (Agilent G1956A SL or VL) for ionization. A 1% formic acid in methanol solution (0.1 ml min^{-1}) was added to provide appropriate pH conditions for positive mode ionization. Peaks previously determined to have hemolytic activity were quantified as a mass (pg) based on calibration curves determined with pure standards. The planar structures, total ion chromatograms and mass spectra of KmTx-1 and KmTx-2 are shown in Fig. 1. This strain produced 2 chromatographically distinct KmTx1 congeners (KmTx 1-1 and KmTx 1-3). Both of these 2 congeners have the same UV absorption maximum at 225 nm, but they have slightly different analytical retention times (Bachvaroff et al. 2008). The relative hemolytic potencies of KmTx1-1 and KmTx1-3 are nearly equivalent but 10 times greater than KmTx 2 (see Bachvaroff et al. 2009). There was no significant effect of P or CO_2 availability on the mass ratio of KmTx 1-1 to KmTx 1-3, which ranged from 0.68 to 0.75 under all of our experimental conditions. Hence, for the calculation of KmTx-1 levels, KmTx1-1 and KmTx1-3 were summed and reported together.

Relative cellular toxicity comparisons. The relative toxicity of the cells in the experimental treatments was compared with a bioassay using the terrestrial plant toxin saponin as a potency standard. Picosaponin cell equivalents were calculated by dividing the saponin concentration responsible for 50% hemolysis of red blood cells by the karlotoxin concentration of each congener (i.e. KmTx 1 and KmTx 2) responsible for 50% hemolysis of red blood cells, and then multiplying this value by the karlotoxin cell quota determined for each treatment (Bachvaroff et al. 2009).

Statistical analysis. Significant differences in growth rates and karlotoxin quotas were determined using a 2-way ANOVA (Zar 1999). Differences between treatment groups were tested by Tukey's Honestly Significant Difference (HSD) multiple comparisons. Differences are termed significant when $p < 0.05$.

RESULTS

Toxin production

In general, most *Karlodinium veneficum* strains only produce one type of toxin, either KmTx-1 or KmTx-2. *K. veneficum* strains isolated from the Chesapeake Bay, USA, have been found to produce exclusively KmTx-1 karlotoxins, while those south of the bay produce exclusively KmTx-2 karlotoxins (Deeds et al. 2004, Place et al. 2008, Bachvaroff et al. 2009). For strain CCMP 2936 (isolated from the Delaware Inland Bays, just north of Chesapeake Bay), both P and CO_2 availability affected not only the level, but also the type of karlotoxin produced. When this strain was grown under P-limited conditions at low CO_2 (230 ppm), it produced predominately KmTx-2, but its toxin phenotype changed to a KmTx-1 type when it was grown under high CO_2 (745 ppm) (Fig. 1). Under normal growth conditions, KmTx-2 production was barely detectable.

The amounts of karlotoxins produced were normalized to chl *a* (Fig. 2), since the cellular chl *a* content remained constant regardless of the availability of pCO_2 and P (data not shown). We considered this preferable to normalization on a per cell basis, since the cell size of some algae can be affected by CO_2 levels (Burns et al. 2005, Levitan et al. 2007) and nutrient availability (Bertilsson et al. 2003). Trends in karlotoxin production normalized to cell numbers were, however, generally similar to those presented here normalized to chl *a* (data not shown).

Increasing pCO_2 stimulated the production of KmTx-1 regardless of P availability, but this effect was pronounced under P-limited conditions ($p = 0.0001$, Fig. 2A). In P-limited cultures, KmTx-1 content at 230 ppm pCO_2 was only 64 and 45% of those in the 430 and 745 ppm treatments, respectively ($p = 0.007$ and $p < 0.0001$). In P-replete cultures, the KmTx-1 toxin contents at a pCO_2 of 230 ppm were close to those at 430 ppm ($p > 0.05$), but there was a significant difference in toxin contents between the 2 higher pCO_2 treatments ($p < 0.05$).

In contrast, the production of KmTx-2 showed the opposite trend, in that there was a significant reduction in KmTx-2 content with increasing pCO_2 regardless of phosphate availability ($p < 0.0001$ and $p = 0.0005$ for P-limited and P-replete treatments, respectively, Fig. 2B). In P-limited cultures, KmTx-2 was dramatically reduced at both elevated pCO_2 relative to the 230 ppm pCO_2 treatment ($p = 0.02$ and $= 0.006$ for 430 and 745 ppm pCO_2 treatments, respectively), and also small but significant differences were observed between the 2 elevated pCO_2 treatments ($p = 0.05$). In contrast to P-limited conditions, KmTx-2 increased modestly in the P-replete 230 ppm pCO_2 cultures over the other 2 enhanced pCO_2 treatments, but this small increase was not statistically significant. The 2 P-replete elevated pCO_2 treatments (430 and 745 ppm) had similar values of toxin production ($p > 0.05$, Fig. 2B).

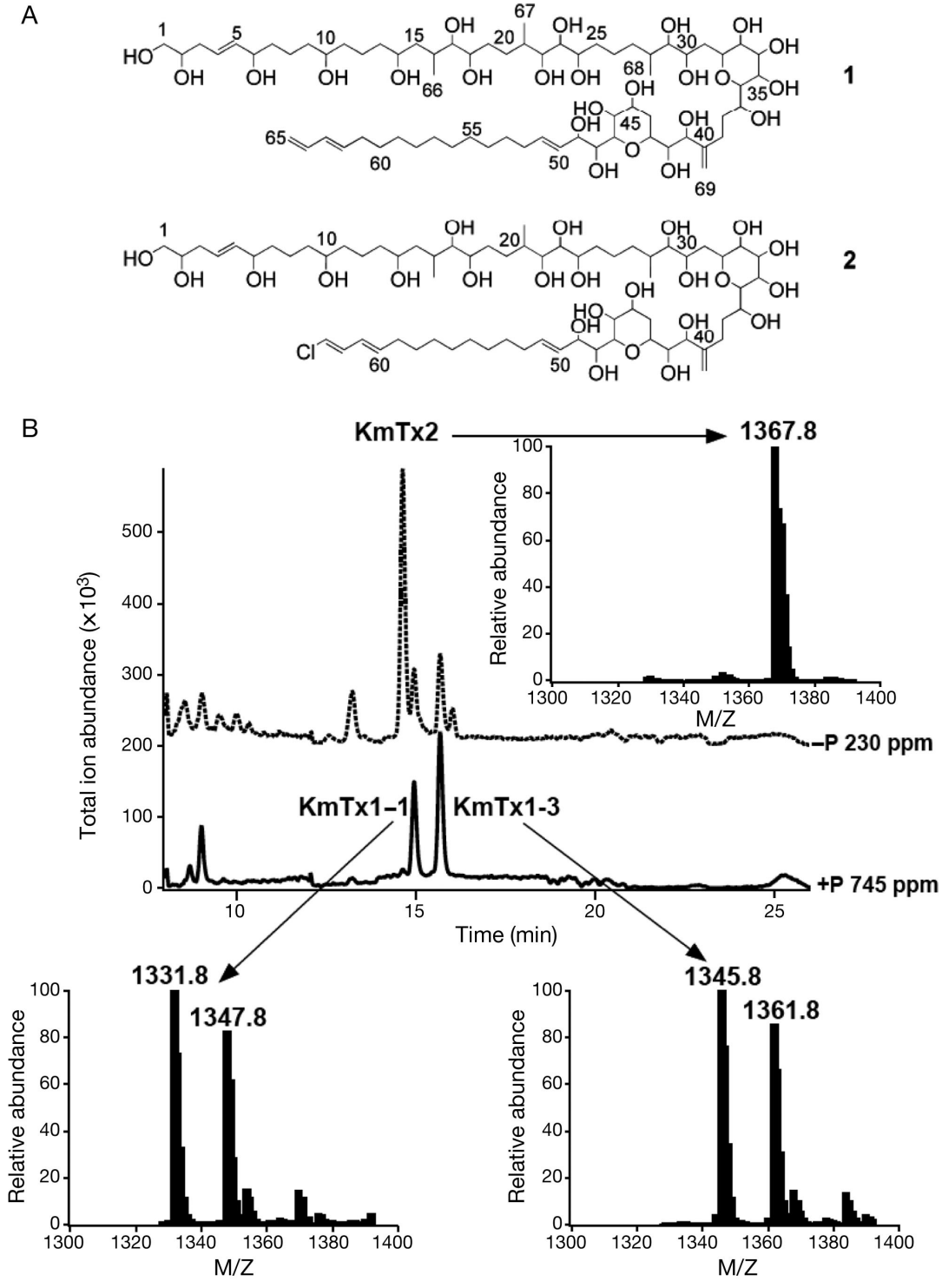

Fig. 1. *Karlodinium veneficum*. (A) Planar structures of KmTx-1-3 (1) and KmTx-2 (2) karlotoxins. The addition of the chlorine atom to the terminal double bond moves the UV absorption maximum from 225 to 235 nm, which is diagnostic for KmTx-2. (B) The total ion chromatogram observed for *K. veneficum* (Center for the Culture of Marine Phytoplankton [CCMP] strain 2936) when P-limited cultures were grown at low (230 ppm) CO_2 (upper trace), or were grown under P-replete and elevated (745 ppm) CO_2 conditions (lower trace). The mass spectra for the dominant peaks under these 2 conditions are shown as insets. Karlotoxins are observed as sodium adducts (+23 amu [atomic mass unit]) under these electrospray conditions. The mass spectra observed for the dominant peak under P-limited and 230 ppm CO_2 conditions are consistent with the KmTx-2 structure shown in (2). During P-replete growth at 745 ppm CO_2 levels, both KmTx-1-1 and KmTx-1-3 are observed along with their 16 amu hydroxylated congeners. The sum of both KmTx 1-1 and KmTx-1-3 is included in results presented for the KmTx-1 toxin. M/Z: mass to charge ratio

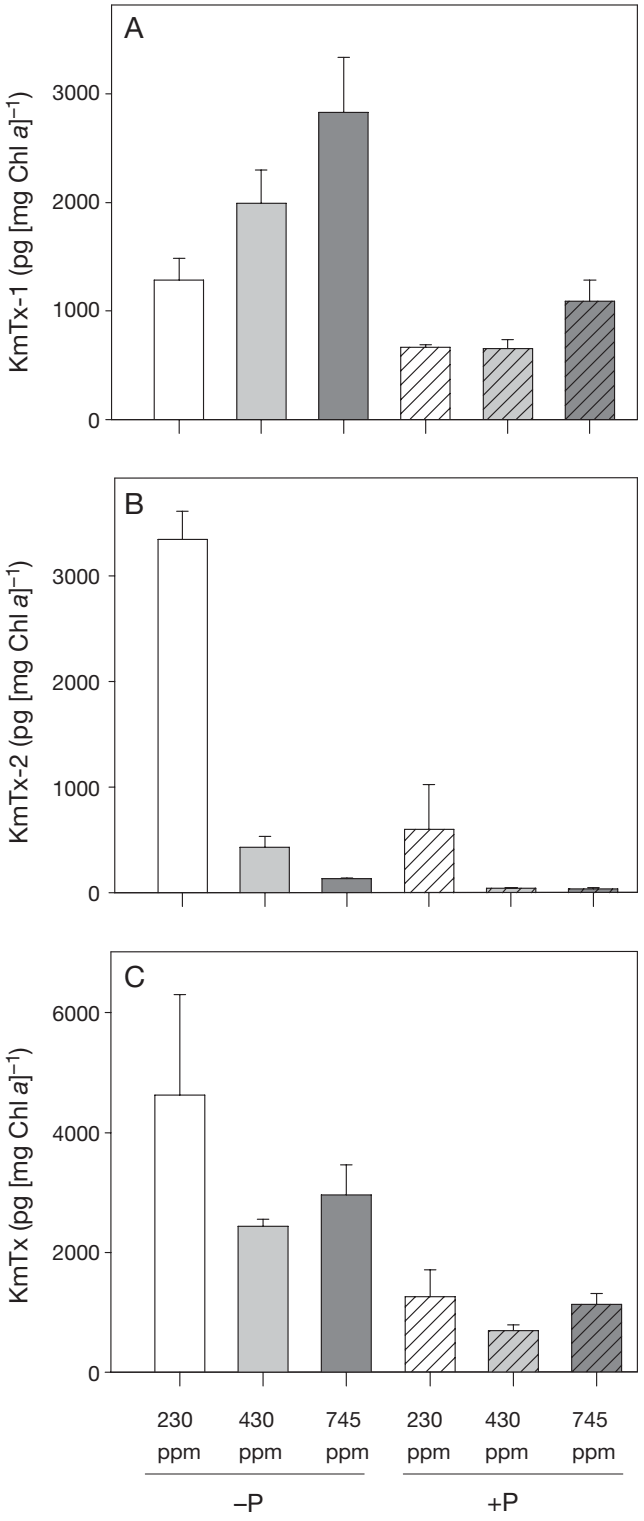

Fig. 2. *Karlodinium veneficum*. Chl *a*-normalized karlotoxin production by *K. veneficum* (Center for the Culture of Marine Phytoplankton [CCMP] strain 2936) in the 6 P and CO_2 treatments. (A) KmTx-1, (B) KmTx-2, (C) total KmTx (KmTx-1 + KmTx-2). –P: P-limited cultures; +P: P-replete treatments; increasing shades of grey represent increasing pCO_2 levels in the treatments. Error bars are SD of triplicate samples

As has been previously reported (Adolf et al. 2009), the toxin cell quotas were much higher in P-limited cultures relative to P-replete ones ($p = 0.0001$). At corresponding pCO_2 levels, in P-limited cultures chl *a*-normalized KmTx-1 contents were 2- to 3.1-fold higher ($p = 0.02$, $p < 0.0001$ and $p < 0.0001$ for 230, 430 and 745 ppm pCO_2 treatments, respectively) and KmTx-2 contents were 3.5- to 10.3-fold higher than those of P-replete cultures. The ratios of KmTx-1 to KmTx-2 in P-limited cultures and P-replete ones ranged from 0.4 to 21 and 2 to 28, respectively (data not shown), largely because KmTx-2 was more sensitive to phosphate availability.

In summary, there was a highly significant interactive effect between pCO_2 and phosphate availability for production of both karlotoxins. KmTx-1 levels were significantly enhanced by the combination of increasing pCO_2 and decreasing P ($p < 0.0001$), and KmTx-2 content was significantly higher with decreasing pCO_2 and decreasing P ($p < 0.001$).

Because CO_2 enrichment had a positive effect on KmTx-1 production and a negative effect on KmTx-2 production at each phosphate condition, there was no significant effect of pCO_2 on the total or summed karlotoxin content ($p > 0.05$, Fig. 2C). However, because of differential potencies of the KmTx-1 and KmTx-2 toxins, overall cellular toxicity was still greatly increased at higher pCO_2 (see 'Discussion'). Total karlotoxin content was elevated 3.6 times (230 ppm pCO_2), 3.5 times (430 ppm pCO_2) and 2.6 times (745 ppm pCO_2) in P-limited cultures compared with P-replete ones at the corresponding pCO_2 levels ($p = 0.003$, 0.01 and 0.01, respectively).

Growth rates

As expected, P-replete cultures grew much faster than P-limited cultures, with growth rates increasing by 140% (230 ppm), 198% (430 ppm) and 229% (745 ppm) under equivalent CO_2 conditions (Fig. 3, $p < 0.0001$). The growth of *Karlodinium* was greatly accelerated by elevated pCO_2 levels in P-replete cultures (Fig. 3A). Growth rates in the 230 ppm pCO_2 treatment were significantly lower than in the other 2 treatments ($p = 0.0002$ and $p < 0.0001$ for 430 and 745 ppm pCO_2 treatments, respectively) and they were also somewhat higher in the 745 ppm CO_2 treatment than in the 430 ppm CO_2 treatment ($p = 0.002$). However, the effect of changing pCO_2 on growth rates of P-limited cultures was minor (Fig. 3A). A slight but significant increase in growth occurred only between the 230 and 745 ppm CO_2 treatments ($p = 0.04$). For the entire data set, KmTx-1 and KmTx-2 toxin levels were most elevated in cul-

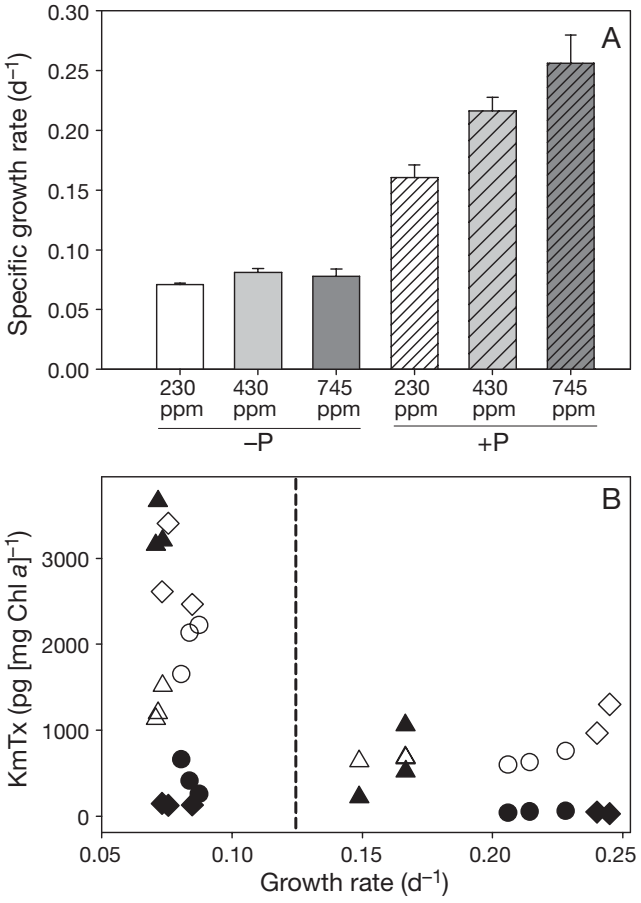

Fig. 3. *Karlodinium veneficum*. (A) Specific growth rates of *K. veneficum* (Center for the Culture of Marine Phytoplankton [CCMP] strain 2936) in the 6 P and CO_2 treatments (symbols and error bars as in Fig. 2). (B) Specific growth rates versus Chl *a*-normalized KmTx-1 and KmTx-2 content. Data plotted on the right and left sides of the dashed lines are from P-replete and P-limited treatments, respectively. Open and closed symbols denote KmTX-1 and KmTX-2, respectively. △▲: 230 ppm pCO_2 treatments; ○●: 430 ppm pCO_2 treatments and ◇◆: 745 ppm pCO_2 treatments

tures whose growth rates were strongly P-limited (<0.075 d^{-1}), and pCO_2 then secondarily controlled the levels of each toxin (Fig. 3B).

DISCUSSION

Karlotoxin production relative to the availability of CO_2 and P

This is the first evidence that toxin production by a dinoflagellate is sensitive to changes in atmospheric CO_2 levels. The pH is known to affect the production of domoic acid (DA) by the diatom *Pseudo-nitzschia* (Lundholm et al. 2004, Trimborn et al. 2008). Laboratory experiments have shown that N and P depletion can enhance karlotoxin production in *Karlodinium veneficum*; however, unlike our observations for altered pCO_2, no change in the type of karlotoxin produced or induction of karlotoxin in nontoxic strains have been observed under nutrient limitation (Adolf et al. 2009). Our study demonstrates a strong linkage between the availability of CO_2 and P, and cellular karlotoxin content.

The effect of higher CO_2 availability strongly favoring KmTx-1 over KmTx-2 production could potentially have large ecological and environmental consequences. Weight-normalized assays of hemolytic activity and ichthyotoxicity have shown that KmTx 1 is about 10 times more potent than KmTx-2 (Bachvaroff et al. 2009). Calculations that combine this differential potency with our KmTx-1 and KmTx-2 production trends show that the relative cellular toxicity of *Karlodinium veneficum* increased dramatically with decreasing P availability and increasing CO_2 levels (Fig. 4). The cells were roughly an order of magnitude more toxic under P-limited conditions in each pCO_2 condition, and within the P-limited treatment the 745 ppm CO_2 cultures were 2.4 times more toxic than the 230 ppm cultures ($p < 0.05$, Fig. 4). P-limited cells in the 745 ppm treatment were also 1.2 times more toxic than those at 430 ppm, although due to variability between replicates this difference was not significant ($p > 0.05$).

It is known that phenotypic and genomic properties of strains may change in culture over time, and that generalizations based on culture studies should consider intra-specific variability (Lakeman et al. 2009).

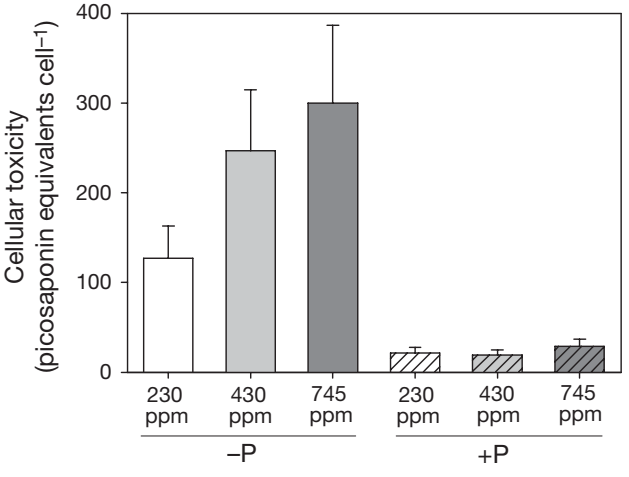

Fig. 4. *Karlodinium veneficum*. Comparison of the karlotoxin cellular toxicity for *K. veneficum* (Center for the Culture of Marine Phytoplankton [CCMP] strain 2936) in the 6 P and CO_2 treatments (symbols and error bars as in Fig. 2). Toxin potency is given in relative units of picosaponin equivalents cell^{-1}

Although the *Karlodinium veneficum* strain we used for these experiments in 2007 was quite recently isolated (2006), we still must acknowledge the limitations of making conclusions about the entire species based on a single strain. Future studies should examine whether karlotoxins are similarly regulated by pCO_2 in a wide selection of recently isolated strains of *Karlodinium*, such as the culture collections examined by Adolf et al. (2009) and Bachvaroff et al. (2009). If it turns out that our results are applicable to other strains of this species, then this raises the possibility that higher future atmospheric pCO_2 levels could produce more toxic and ecologically damaging blooms of *K. veneficum,* especially in those estuaries where the dinoflagellate is also limited by P.

The biochemical mechanism by which CO_2 availability and/or pH affect toxin production remains to be determined. Enzymatic processes, carbon limitation, pH-mediated changes in metal speciation and associated bacterial community composition have all been suggested to affect the production of domoic acid by *Pseudo-nitzschia* (Lundholm et al. 2004), and similar effects may be the case for *Karlodinium.* Although, as with all other complex algal toxins, the biochemical synthetic pathways for karlotoxins have not been fully elucidated, it is evident from our results that the enzymatic bifurcation point for KmTx-1 or KmTx-2 synthesis is sensitive to changing pCO_2 or pH. An influence of external pH on intracellular pH has been observed in some dinoflagellates (Dason & Colman 2004). It has been suggested that there are particular optimum pH values for domoic acid production (Lundholm et al. 2004). Likewise, the synthesis of KmTx-1 and KmTx-2 may also exhibit differential pH effects.

These CO_2-mediated variations in toxin production could potentially be an indirect effect resulting from alleviation of C limitation of carbon fixation. Carbon fixation and toxin production are both energetically expensive processes. Consequently, one possibility is that elevated CO_2 could provide a photosynthetic subsidy that enables diversion of increased amounts of energy and/or carbon to support toxin production. We calculated the fraction of total cell carbon a *Karlodinium* cell must contribute to its cellular karlotoxin quota using the measured levels of toxins in our cultures, the molecular weights of KmTx-1 and KmTx-2, and the measured particulate organic carbon in the samples (data not shown). This calculation suggests that only a relatively small amount of the cellular carbon quota is present as total karlotoxin, regardless of CO_2 and P growth conditions (0.5 to 2.5% of total cellular carbon). Thus, the fraction of photosynthetically derived carbon being used in toxin production appears to be relatively insignificant, suggesting that differences in allocation of fixed carbon are probably not the cause of the observed effect. In addition, if either energy or carbon limitation were to directly control toxin levels in *Karlodinium*, we would expect to see an enhancement in both karlotoxin-1 and karlotoxin-2 at higher levels of CO_2, which was not the case.

Currently, no information is available on the CCM capabilities of *Karlodinium,* so potential physiological linkages between changes in photosynthetic carbon acquisition and toxin production must necessarily remain speculative at the present time. The influences of pH and DIC availability are not necessarily mutually exclusive, and a combination of both could be responsible for the observed toxin production effects. Our study demonstrating the effects of realistically changing carbonate buffer system parameters on cell toxicity should be followed by mechanistic studies in the future to distinguish the individual effects of pH and DIC, such as the 'pH drift' experiments of Hansen et al. (2007). Such experiments are very informative in terms of algal physiology, despite their limited ecological relevance. In natural marine ecosystems, changing pCO_2 necessarily changes pH and vice versa, unless the carbonate system is grossly manipulated. Future work should include both types of experiments, since both cellular physiological pathways and their net effects on ecological processes in the environment are important to understand.

The growth rates of the cultures were generally also affected by both CO_2 and P availability, but significant CO_2 effects were seen only in P-replete cultures. These results suggest that the growth of *Karlodinium* may be limited by DIC as long as it is not nutrient limited. This interaction is a classic example of Liebig limitation, in which the second 'nutrient' (in this case, DIC) can become limiting only when the primary limitation by P is relieved. Thus, this DIC/P interaction fundamentally differs from the co-limitation by both DIC and P observed in the cyanobacterium, *Trichodesmium*, by Hutchins et al. (2007). Growth rates of harmful *Karlodinium* blooms, therefore, may benefit from future increases in CO_2, but possibly only when nutrients are in excess. This contrasts with the toxin results discussed above, in which the biochemical composition of the cellular karlotoxin pool changed with altered pCO_2, especially in P-limited cells. These results suggest a complex relationship between pCO_2, P availability and cellular growth and toxicity in this species.

Previous studies have suggested that in general, phytoplankton toxins will be expressed when the growth rates of phytoplankton are limited (Johansson & Granelli 1999). A connection between P-limitation and enhanced cell toxicity has been observed in *Karlodinium veneficum* (Vaque et al. 2006, Adolf et al. 2009), *Alexandrium fundyense* (John & Flynn 2000) and *Protogonyaulax* (now *Alexandrium*) *tamarensis*

(Boyer et al. 1987). The common explanation for this observation is that by negatively affecting grazers and competing phytoplankton, toxin production under nutrient-limited conditions allows these dinoflagellates to compensate for their lower nutrient uptake efficiencies compared with other phytoplankton groups (Yamamoto & Tarutani 1996, 1999, Smayda 1997). Toxin production may also serve to redirect grazing pressure towards other non-toxic phytoplankton (Guisande et al. 2002). When we plotted KmTx-1 and KmTx-2 toxin levels presented above versus specific growth rates of our cultures (Fig. 3B), the highest levels of toxin production for the entire data set of this isolate were only observed below a threshold growth rate of about 0.075 d^{-1}, which seems to corroborate this growth limitation hypothesis. However, one must also consider the role karlotoxin plays in prey capture. Most strains of *K. veneficum* exhibit significant growth enhancement (2- to 3-fold) when feeding mixotrophically. Nontoxic strains are incapable of enhancing their growth potential through feeding when prey like cryptophytes are available (Adolf et al. 2008).

The karlotoxin cell quotas we observed in this study (P-replete: 1.34 ± 0.12 pg $cell^{-1}$; P-limited: 13.59 ± 1.16 pg $cell^{-1}$) for CCMP 2936 exceed the average values reported for other strains under nutrient-limited growth conditions (Adolf et al. 2009). In general, most strains of *Karlodinium* only produce a single toxin type (i.e. either KmTx-1 or KmTx-2, Bachvaroff et al. 2009), but mixed karlotoxin production (i.e. both KmTx-1 and KmTx-2 types) has been observed in the type species for *K. veneficum*, Plymouth 103 (Galimany et al. 2008). This result raises the question of how those strains that only produce either KmTx-1 or KmTx-2 will respond to changing pCO_2.

Since our study revealed that the availability of P and CO_2 had a large effect on the production of karlotoxin, an important question is whether these results are relevant in the estuaries where harmful *Karlodinium venificum* blooms occur. In the Delaware Inland Bays where our culture was isolated, P limitation often occurs in the upper portion of Indian River Bay (IRB) due to elevated nitrogen inputs from the Indian River (Valdes 2003). During recent summers, a series of harmful algal blooms, including both dinoflagellates and raphidophytes, have been observed in numerous localities throughout the bays. In the IRB, dissolved phosphate concentrations in these recent *Karlodinium* blooms were low (~0.3 µM) and dissolved inorganic N:P ratios were ~100:1 (data not shown). In these cases *Karlodinium* was apparently growing under severely P-limited conditions, so toxin production would be potentially stimulated and also subject to strong control by pCO_2. *K. veneficum* is a mixotroph that is capable of feeding on cryptophyte species, which could potentially provide an alternate source of P in P-limited environments. It is known that nutrient conditions are an important aspect of *K. veneficum* bloom formation in eutrophic environments, and also that prey availability is another factor leading to toxic *K. veneficum* blooms (Adolf et al. 2008). Future studies will be needed to determine the interactions of nutrient limitation, prey and CO_2 availability on *Karlodinium* toxin production.

Our experiments were done with a strain of *Karlodinium veneficum* adapted to relatively high salinity. The salinity used in this study (~25) was chosen to be similar to the portion of the Delaware Inland Bays where this strain was isolated. Although many other strains grow best at salinities of about 15, Adolf et al. (2009) showed that growth rates increased with increasing salinity when the cultures were grown at temperatures above 25°C. It would be interesting to do similar experiments with a strain adapted to low salinity, such as one from the Chesapeake Bay, since changes in pCO_2 should have a more pronounced effect on pH and DIC in lower salinity estuaries. Consequently, it is possible that the effects of pCO_2 on potency and growth would be more pronounced at salinities of 15 or lower.

There are little or no data available on current pH and DIC concentrations in the parts of either the Delaware Inland Bays or in the Chesapeake Bay where *Karlodinium* blooms occur, but typical present day DIC concentrations in nutrient-enriched estuarine waters at salinities of 20 to 27 may range from 1.8 to 2.0 mM (Thomas & Schneider 1999), and blooms of phytoplankton may result in pH values of up to 8.7 in coastal waters (Hinga 2002). These total DIC levels are roughly equivalent to the CO_2 levels of 230 to 430 ppm in our experiment, assuming that temperature and salinity are similar to those used here. Unfortunately, there are no simultaneous field measurements of karlotoxin activity, DIC and phosphate yet to confirm our culture observations that karlotoxin production is subject to the availability of both P and CO_2.

Further work will be needed to determine whether toxin production by other groups of toxic algae could also be regulated by changing CO_2. Equally important is to determine whether their responses to rising CO_2 may be modulated by other environmental factors such as changes in nutrient availability, irradiance and temperature. This is information that will be critically needed to establish the ecological, environmental and economic consequences of harmful algal bloom toxin production in a changing ocean.

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