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Temperature controls the toxicity of the ichthyotoxic dinoflagellate *Cochlodinium polykrikoides*

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ABSTRACT: Blooms of the harmful dinoflagellate *Cochlodinium polykrikoides* occur across temperate and tropical zones of the Northern Hemisphere and have lethal impacts on marine life, including fish, coral, and shellfish. We report a series of laboratory- and ecosystem-based experiments examining the effects of temperature on the growth and lethal effects of *C. polykrikoides*. Growth rates of this alga were positive from 15 to 30°C and were maximal at 24 to 27°C, with cultures cultivated at warmer temperatures (>20°C) yielding significantly higher percentages of cells in chains. In contrast to growth, lethality of clonal isolates and bloom populations of *C. polykrikoides* to both larval fish and larval shellfish significantly decreased as temperatures progressively increased from 16 to 28°C. Clonal populations comprising primarily single cells were significantly more toxic than cell-equivalent densities of populations in chains at the same temperature, suggesting temperature-induced changes in cell concatenation influenced lethal effects. The enzyme catalase was capable of mitigating the lethality of *C. polykrikoides* to fish at higher, but not lower, temperatures, suggesting that higher rates of enzyme activity by the alga and/or target organisms at higher temperatures may mitigate toxicity associated with reactive oxygen species. Collectively, these findings suggest that (1) as blooms in temperate zones progress from summer to fall, their lethality may increase, (2) blooms occurring at tropical latitudes may be less toxic than those in temperate regions, and (3) as global temperatures increase, *Cochlodinium* spp. blooms may expand into new, higher-latitude regions, where they may be highly lethal.

KEY WORDS: *Cochlodinium polykrikoides* · Climate change · Reactive oxygen species · Harmful algal blooms · *Mercenaria mercenaria* · *Argopecten irradians* · *Menidia beryllina* · *Cyprinodon variegatus*

INTRODUCTION

Climate change is predicted to alter the dynamics and occurrence of harmful algal blooms (HABs) (Paerl & Huisman 2008, Hallegraeff 2010, Fu et al. 2012, Hinder et al. 2012, Glibert et al. 2014). Temperature, in particular, is expected to have strong impacts on the distribution, timing, and proliferation of HABs (Hallegraeff 2010, Fu et al. 2012). For example, Moore et al. (2008) reported that a future sea surface temperature increase of 2°C could double the window of opportunity for blooms of the toxic dinoflagellate *Alexandrium catenella* to occur in Puget Sound, WA, USA. Growth of toxic cyanobacterial species is favored over non-harmful, eukaryotic phytoplankton at higher temperatures (Paerl & Huisman 2008). Future, warmer oceans are expected to experience stronger stratification that will decrease inorganic nutrient availability (Behrenfeld et al. 2006, Doney et al. 2012), conditions that would favor HABs that are typically capable of diel vertical migration (Smayda & Reynolds 2003) and/or mixotrophy (Burkholder et al. 2008, Heisler et al. 2008).

Some HABs are also expected to exhibit enhanced toxin production under a variety of climate change scenarios, including enhanced stratification/nutrient-

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limiting conditions and ocean acidification (Hallegraeff 2010, Tatters et al. 2012, Hattenrath-Lehmann et al. 2015). However, the relationship between HAB toxicity and temperature change is less clear. For example, Anderson et al. (1990) reported increased saxitoxin production by *Alexandrium fundyense* and *A. catenella* cultivated at 8°C compared to 15°C, whereas others described higher toxin production by *A. fundyense* at 25°C compared to 15°C (Etheridge & Roesler 2005). Regarding diarrhetic shellfish toxins (DSTs), Tong et al. (2011) observed sustained high DST production by *Dinophysis acuminata* at low temperatures (4 and 6°C), while others reported increased toxin production, coupled with increased growth rates, at warmer temperatures (14 to 22°C; Kamiyama et al. 2010). Although all of these studies measured cellular toxin content in response to temperature, none considered the subsequent effects of those toxins on organisms in temperature-altered environments.

While some HABs synthesize potent neuro- or gastrointestinal toxins that target a broad range of organisms, including humans, many other ecosystem-disruptive algal blooms can cause widespread and rapid mortality in fish populations, but are harmless to humans (Sunda et al. 2006). The mechanisms by which algae such as *Chattonella* spp., *Heterosigma* spp., *Fibrocapsa* spp., *Akashiwo sanguinea*, and *Cochlodinium* spp. (Oda et al. 1997, Kim et al. 1999, Kim et al. 2002, Marshall et al. 2005, Tang & Gobler 2009a, Hallegraeff 2010) are ichthyotoxic is not well understood, although in many cases toxicity may be related to the production of reactive oxygen species (ROS) (Ishimatsu et al. 1996, Oda et al. 1997, Kim et al. 1999, Marshall et al. 2005, Tang & Gobler 2009a). Given the temperature dependence of ROS production in some photosynthetic organisms (Yu 1994, Gill & Tuteja 2010) and potential temperature effects on ROS solubility and/or scavenging (Wise 1995, Miller et al. 2010), the lethality of these ichthyotoxic algae could be temperature-dependent.

One of the most cosmopolitan genera of ichthyotoxic algae is *Cochlodinium*. It is globally distributed across the Northern Hemisphere and has caused widespread damage to wild and aquacultured populations of fish, coral, and shellfish in North America and Asia (Kim et al. 1999, Gobler et al. 2008, Kudela et al. 2008, Kudela & Gobler 2012). To date, multiple studies have assessed the role of temperature in the occurrence of *Cochlodinium* blooms. Kim (2004) reported that east Asian strains of *Cochlodinium polykrikoides* exhibited maximal growth at warmer temperatures (~25°C). Similar thermal tolerances for *C. polykrikoides* are reported for blooms occurring on the east coast of the USA (Gobler et al. 2008, Mulholland et al. 2009, Kudela & Gobler 2012). In contrast, *C. polykrikoides* blooms in tropical and subtropical regions such as the Arabian Gulf (Richlen et al. 2010), Malaysia (Anton et al. 2008), and the Philippines (Azanza et al. 2008) have occurred at significantly warmer temperatures (27 to 36°C). Despite this range in temperature tolerance, the effect of temperature on the toxicity of *Cochlodinium* spp. and other fish-killing algae is unknown.

Here we report on the influence of temperature on the growth and toxicity of North Atlantic strains and natural blooms of *C. polykrikoides* (Malaysian–American ribotype; see Iwataki et al. 2008). Clonal isolates were cultivated for multiple generations along a temperature gradient (15 to 33°C) to determine the relationship between temperature and growth. In addition, a series of toxicity experiments were conducted at multiple temperatures whereby multiple early-life-stage marine organisms were challenged with both clonal and wild populations of *C. polykrikoides* to identify the impacts of temperature on the toxicity of this alga.

MATERIALS AND METHODS

Growth experiments

Cultures of *Cochlodinium polykrikoides* (strains CP-1 and CPSB-1G), isolated from Flanders Bay and Noyac Bay, NY, USA in 2006 and 2009, respectively (Gobler et al. 2008, Tang & Gobler 2010), were grown in sterile GSe medium (Doblin et al. 1999) at a salinity of 32 to 33 with antibiotics (1% v/v final concentration; 10 000 IU penicillin, 10 000 µg ml^{-1} streptomycin, 25 mg ml^{-1}, amphotericin B) to minimize bacterial contamination. Stock cultures were maintained at 21°C on a 12 h light:12 h dark cycle with a light intensity of ~100 µmol quanta m^{-2} s^{-1}. Prior to experiments, a large volume (~3 l) batch culture of *C. polykrikoides* was prepared in sterile GSe medium and allowed to reach exponential growth, serving as inoculum for temperature–growth experiments. Smaller flasks (250 ml) were prepared with sterile GSe and cultures were diluted to achieve an initial cell density of ~700 cells ml^{-1}. Triplicate flasks were placed into a thermal gradient chamber (TGC; modified from Blankley & Lewin 1976) at temperatures of 15, 18, 21, 24, 27, 30, and 33°C, which varied by no more than 1% of targeted temperatures during experiments. Cultures were monitored through ex-

ponential growth phase, transferred into new media during late exponential growth phase, and aliquots were preserved in Lugol's iodine for microscopic cell enumeration during each transfer. Cultures were grown at each temperature for >10 generations and growth rates (μ) in doublings d^{-1} were calculated as $\mu = [\ln(B_0 - B_1)/t]/\ln(2)$, where B_0 is the initial cell density, B_1 is the final cell density, and t is the period of time cultures were allowed to incubate. Given that growth rates generally stabilized at each temperature after 5 generations, reported growth rates are based on the mean across the final 5 generations at each temperature. The percentage of cells in chains of various lengths (1 to 4 cells) was also quantified at each temperature via microscopy.

Larval fish

Larval *Menidia beryllina* (inland silverside) and *Cyprinodon variegatus* (sheepshead minnow) were obtained from >100 broodstock fish maintained at Aquatic Research Organisms (Hampton, NH, USA) and the Stony Brook-Southampton Marine Sciences Center (broodstock collected from Shinnecock Bay, NY, USA). Larvae were maintained in flow-through, recirculated seawater at ~22°C and fed a diet of live *Artemia salina* (brine shrimp) daily until experimentation. All fish larvae used in experiments were less than 1 wk old and 2 to 4 mm in length (Table 1). Temperature-controlled water baths at 16, 20, 24, and 28°C were established for experiments via use of either aquarium chillers (IceProbe; Nova Tec) or heaters (25 W; Hydor USA) with light levels of 100 μmol quanta m^{-2} s^{-1} (Table 1). Separate *C. polykrikoides* cultures were prepared for each trial and were grown in flasks with fresh media for at least 5 d at each temperature to ensure cells were in exponential phase growth for experiments. To commence experiments, cultures at each temperature were transferred to sterile, polystyrene, 6-well, 10 ml culture plates at cell densities that ranged from 500 to 2000 cells ml^{-1} (achieved via dilution with GSe media; Table 1), representing moderate to dense blooms (Kudela & Gobler 2012) and abundances shown to elicit lethal effects on fish (Tang & Gobler 2009a). One 6-well plate, as each plate yields 6 experimental replicates, was established at each combination of temperature and cell density, and a no-algae control treatment of sterile GSe medium was also established at each temperature. Replicate plates (n = 3) were included for each treatment in preliminary trials and yielded results nearly identical to trials with only 1 plate. Therefore, in subsequent experiments, replicate plates were not used to limit the number of fish sacrificed. Accordingly, larval fish were placed into each well (n = 1 fish per well, 6 fish per plate) using a modified transfer pipette, and an antibiotic solution (1% v/v as described above) was added to discourage bacterial contamination (Rountos et al. 2014). Fish were examined several times throughout the exposure period (24 to 72 h) to assess mortality. Dead fish were characterized by a failure to maintain a vertical position, lack of horizontal movement, lack of gill movement, and failure to respond to external stimulation. At the end of experiments, plates were preserved with a Lugol's iodine solution (1% v/v) and final cell densities were determined via light microscopy.

Bloom experiments

Water was collected from blooms of *C. polykrikoides* in Old Fort Pond (40.868° N, 72.446° W)

Table 1. Conditions for all experiments. *Mercenaria mercenaria* embryos were less than 4 h old, *Argopecten irradians* veligers were ~10 d old, and all fish (*Cyprinodon variegatus*, *Menidia beryllina*) were less than 1 wk old

Trial	Species	Exposure type	Temp(s). (°C)	Exposure densities (cells ml^{-1})	Exposure time (h)	Mean size ± SD (mm) or stage
1	*Cyprinodon variegatus*	Culture (CP-1)	16, 20, 24, 28	0, 850, 1900	48	3.77 ± 0.74
2	*C. variegatus*	Culture (CPSB-1G)	16, 20, 24, 28	0, 500	24	2.45 ± 0.59
3	*Menidia beryllina*	Culture (CP-1)	16, 20, 24, 28	0, 500, 1000	72	2.73 ± 0.42
4	*Mercenaria mercenaria*	Culture (CP-1)	20, 24, 28	0, 600	24	Embryo
5	*Argopecten irradians*	Culture (CP-1)	16, 20, 24, 28	0, 500, 1000, 2000	24	Veliger
6	*C. variegatus*	Bloom	19, 23, 25, 28	5000	24	4.10 ± 0.61
7	*M. beryllina*	Bloom	19, 23, 25, 28	250	24	4.07 ± 0.37
8	*M. beryllina*	Short vs. long chain (CP-1)	21	0, 1000	48	2.81 ± 0.35
9	*M. beryllina*	Catalase (CP-1)	21	0, 2000	5	2.81 ± 0.35

within eastern Shinnecock Bay, NY, USA, during September 2014 (water temperature ~22°C). Bloom water was collected from ~0.25 m depth in 20 l carboys and transported back to the Stony Brook University-Southampton Marine Sciences Center. Within <2 h of water collection, exposures of larval fish (*C. variegatus* and *M. beryllina*) to the bloom water were conducted at temperatures of 19, 23, 25, and 28°C using the identical experimental design to that described above (see Table 1). Filtered (0.2 µm) bloom water was used as a control treatment (Tang & Gobler 2009a). Mortality was noted after 24 h of exposure.

Bivalve larvae

Larvae from *Mercenaria mercenaria* (hard clam, northern quahog) and *Argopecten irradians* (bay scallop) were obtained from the Stony Brook University-Southampton Shellfish Hatchery (Southampton, NY, USA) and the East Hampton Town Shellfish Hatchery (Montauk, NY, USA) via thermal stimulation (~28°C). *A. irradians* broodstock were collected from eastern Shinnecock Bay and spawned within 2 h of collection. Larvae were reared in conical tanks (~200 l) with mild aeration at 24°C and fed a diet of live cultured *Isochrysis galbana* (Tahitian strain; T-Iso) until use for experiments at 10 d post-fertilization. *M. mercenaria* embryos, provided by the East Hampton Town Shellfish Hatchery, were obtained from broodstock collected from the eastern Peconic Estuary. Exposures with embryos were conducted within 4 h of fertilization.

Experiments were established in 6-well plates containing varying densities of *C. polykrikoides* (CP-1) pre-adapted to ~16, 20, 24, and 28°C as described above for larval fish (see Table 1 for densities and temperatures), with GSe media serving as a control at each temperature. A total of 5 *M. mercenaria* embryos or *A. irradians* veligers were transferred to each 10 ml well of a 6-well culture plate, resulting in densities consistent with field observations in US east coast estuaries (Carriker 1961). Survival of *M. mercenaria* embryos and activity of *A. irradians* veligers were examined under a dissecting microscope after 24 h. Embryos developing into trochophores and/or straight-hinged veligers and actively swimming were identified as viable. To assess *A. irradians* veliger activity, wells containing larvae were gently stirred, allowed to sit undisturbed for 1 min, and examined. The number of veligers actively swimming and maintaining a vertical position in the water were identified as active as compared to veligers that were completely immobile, but alive and exhibiting internal movement, lying at the bottom of plates. All plates were preserved with a Lugol's iodine solution (1% v/v) at the end of experiments and final *C. polykrikoides* densities were determined via light microscopy.

Chain length experiments

To assess the differential lethality of cells in chains compared to single cells, an experiment was conducted whereby larval silversides *M. beryllina* were exposed to cultures with a large and small proportion of cells in chains of 4. A stock culture grown at 21°C was gently poured over a 10 µm mesh to remove longer assemblages of cells. Cells retained on the mesh were immediately rinsed with sterile filtered (0.2 µm) seawater into sterile GSe media. An experiment was subsequently conducted in which *M. beryllina* larvae were exposed to 1000 cells ml^{-1} of sieved culture (containing 75% single cells and 25% chains of 2 cells) and the culture retained on the sieve (30% single cells, 35% chains of 2, and 35% chains of 4). Exposures were conducted as described above at ~21°C. In addition to fish survival, activity of cells from short and long chain fractions was confirmed in plates to ensure viability of cells throughout the exposure.

Catalase experiments

To investigate the impacts of temperature on the potential enzymatic neutralization of ROS, fish bioassays with *M. beryllina* were conducted at high and low temperatures, with and without the addition of catalase, a ROS-scavenging enzyme that has previously been shown to mitigate lethal effects of *C. polykrikoides* on fish (Tang & Gobler 2009a). Treatments were specifically established using 2000 cells ml^{-1} at 16 and 28°C, with and without the addition of 2 U catalase ml^{-1}. The experiment was conducted as described above and the status of the fish (live or dead) was monitored continuously for the duration of the experiment (~4 h).

Statistics

Statistical analyses were performed using Rstudio statistical software (CRAN R, version 3.1.0; www.R-

project.org). Growth rate and relative abundances of different chain lengths of *C. polykrikoides* across gradients of temperature were analyzed using a 1-way analysis of variance (ANOVA). All proportion data analyzed via ANOVA were arcsine square-root transformed prior to analysis. Assumptions of normality and homoscedasticity were confirmed using a Shapiro-Wilks test for normal distribution and Levene's test for homogeneity of variance. When significant differences were detected, a Tukey's honest significant difference test (Tukey's HSD) was performed. Survival of fish exposed to *C. polykrikoides* across various temperatures was analyzed via Kaplan-Meier survival analysis (Efron 1988, Crawley 2013), with time to death compared for each treatment using the survival package (http://CRAN.R-project.org/package=survival). The proportion of fish surviving beyond 24 h exposure to bloom populations of *C. polykrikoides* was analyzed using Marascuilo's procedure for comparison of multiple proportions (Marascuilo 1966). Replicates (n = 5) within exposure plate wells allowed for shellfish responses to be analyzed using standard ANOVAs. As such, mortality of *M. mercenaria* embryos was analyzed using Welch's *t*-tests to assess the effects of exposure at each temperature. Because *A. irradians* veliger activities did not conform to a normal distribution, a Kruskal-Wallis ANOVA followed by a Nemenyi post hoc test was conducted to assess differences in activity among levels of cell densities within each temperature. All results were deemed significant at $\alpha \leq 0.05$ ($p < 0.05$).

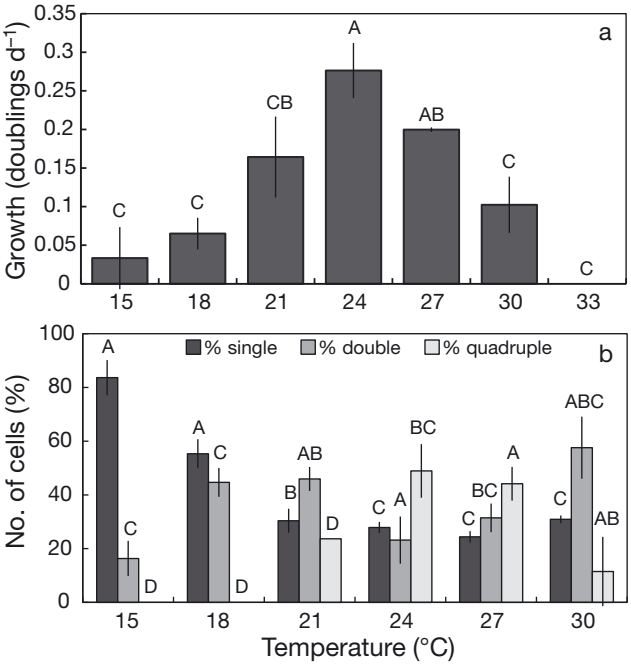

Fig. 1. Effect of temperature on (a) growth and (b) composition of *Cochlodinium polykrikoides* clone (CP-1). Error bars represent ± SD; letters denote significant groupings; $p < 0.05$ (1-way ANOVA)

RESULTS

Growth rate experiments

Growth rates of *Cochlodinium polykrikoides* (clone CP-1) were significantly affected by temperature ($p < 0.001$; ANOVA; Fig. 1a). Maximal growth occurred at 24°C (0.27 doublings d^{-1}), while growth was significantly lower at temperatures below 18°C and at those exceeding 27°C ($p < 0.05$; Tukey's HSD). Cells did not grow at the lower and upper thresholds of <15°C (data not shown) and 33°C, respectively (Fig. 1a). Chain length of *C. polykrikoides* was dependent upon temperature, with the abundance of cells in chains (>2 cells) increasing with temperature ($p < 0.05$; ANOVA; Fig. 1b). At colder temperature treatments (15 and 18°C), the abundance of single cells was significantly greater than at warmer temperatures, whereas cells in chains of 2 significantly increased at temperatures of 18°C and above ($p < 0.05$; Tukey's HSD; Fig. 1b). Chains of 4 cells were present only at warmer temperatures (21 to 30°C). Paralleling growth, the greatest abundance of 4-cell chains occurred at 24°C and declined thereafter.

Fish exposures

Sheepshead minnows *Cyprinodon variegatus*

Both species of fish exposed to *C. polykrikoides* were highly sensitive to this alga, with time to death in colder temperature treatments being significantly more rapid compared to warmer treatments. Final survival and time to death were both dose- and temperature-dependent. Mortality rates of sheepshead minnows *C. variegatus* exposed to 1900 cells ml^{-1} at 16 and 20°C were significantly faster than fish at 24 and 28°C ($p < 0.001$; Kaplan-Meier survival analysis; Fig. 2a). All fish at 16 and 20°C (1900 cells ml^{-1}) died within 1 h, whereas fish at 24 and 28°C displayed 50 and 100% survival after 48 h, respectively ($p < 0.001$; Kaplan-Meier survival analysis; Fig. 2a). A similar pattern was observed for *C. variegatus* exposed to 850 cells ml^{-1} of *C. polykrikoides,* whereby all fish at

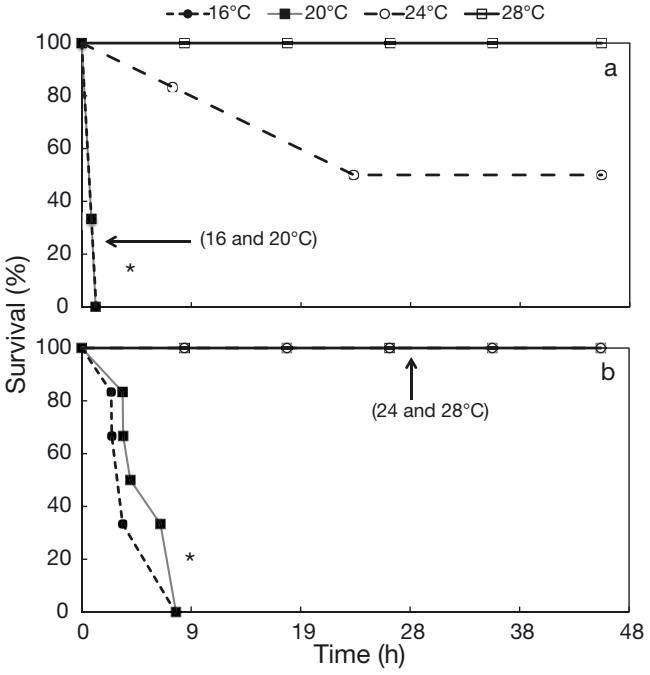

Fig. 2. Survival of *Cyprinodon variegatus* exposed to (a) 1900 and (b) 850 cells ml^{-1} at 16, 20, 24 and 28°C. There was no mortality observed in control treatments (data not shown). Time to death in colder temperature treatments was significantly hastened. *p < 0.001 (Kaplan-Meier survival analysis)

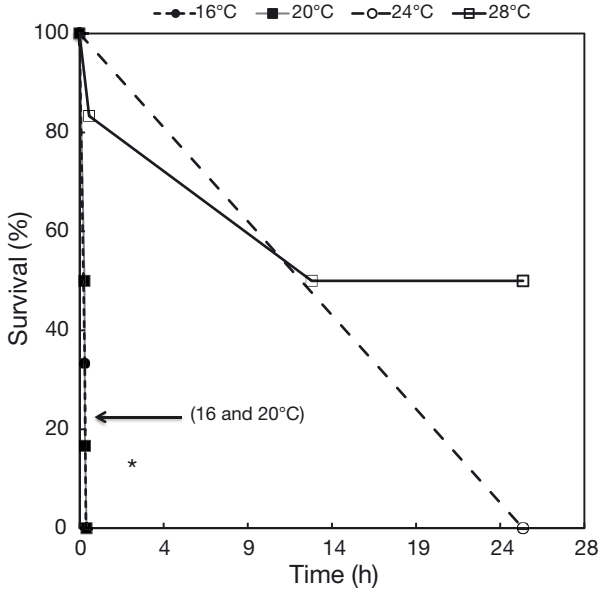

Fig. 3. Survival of *Cyprinodon variegatus* after 24 h exposed to 500 cells ml^{-1} of *Cochlodinium polykrikoides* (CPSB-1G) at 16, 20, 24, 28°C. There was no mortality in control treatments at any temperature (data not shown). Survival of fish at 16 and 20°C was significantly reduced compared to 24 and 28°C treatments. *p < 0.001 (Kaplan-Meier survival analysis)

16 and 20°C died within 8 h, and fish exposed at 24 and 28°C survived until the end of the experiment (48 h; Fig. 2b). No mortality was observed in any control treatment fish (0 cells ml^{-1}). To ascertain whether patterns of toxicity were strain-specific, a separate experiment was conducted with an additional strain of *C. polykrikoides* (CPSB-1G) at a density of 500 cells ml^{-1}. In this trial, toxicity was, again, inversely related to temperature. Time to death of *C. variegatus* was significantly accelerated in colder temperature treatments (16 and 20°C) compared to higher temperatures (24 and 28°C; $p < 0.001$; Kaplan-Meier survival analysis; Fig. 3).

Inland silversides *Menidia beryllina*

Time to death of inland silversides *M. beryllina* was also significantly more rapid in fish exposed to *C. polykrikoides* (CP-1) at colder temperatures, and was dose-dependent (Fig. 4). When exposed to 1000 cells ml^{-1} of *C. polykrikoides*, time to death (20 h) was significantly faster at 16 and 20°C compared to 24 and 28°C (~67 h; $p < 0.001$; Kaplan-Meier survival analysis; Fig. 4a). At lower cell densities (500 cells ml^{-1}), all fish exposed to *C. polykrikoides* at 16°C perished

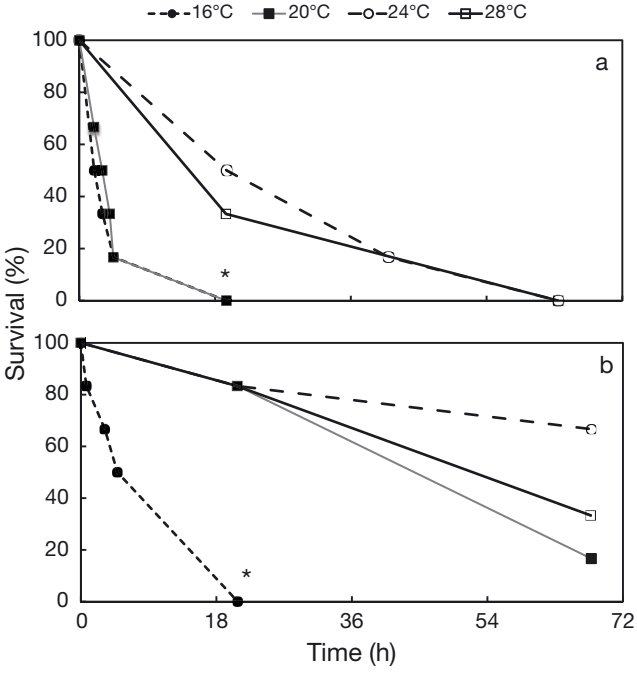

Fig. 4. Survival of *Menidia beryllina* exposed to (a) 1000 and (b) 500 cells ml^{-1} CP-1 at 16, 20, 24, 28°C. There was no mortality in control treatments (data not shown). Survival was significantly reduced in colder temperature treatments. *p < 0.001 (Kaplan-Meier survival analysis)

within 20 h, whereas a proportion of fish at 20, 24, and 28°C survived the duration of the experiment (>67 h; $p < 0.001$; Kaplan-Meier survival analysis; Fig. 4b). No mortality was noted in any of the control fish at any temperature during experiments with *M. beryllina*.

Bivalve larvae exposures to clonal isolates

Hard clam *Mercenaria mercenaria* embryos were sensitive to *C. polykrikoides* (600 cells ml^{-1}), but only at 20°C (Fig. 5). After 24 h, mortality of embryos exposed to *C. polykrikoides* at 20°C was 100%, and significantly elevated relative to control treatments ($p < 0.05$; Welch's *t*-test; Fig. 5). There was no effect of *C. polykrikoides* on the survival of embryos exposed at warmer temperatures (24 and 28°C; Fig. 5).

The ability of *C. polykrikoides* to render *Argopecten irradians* veligers (~1 wk old) immobile was both temperature- and dose-dependent. At the lowest exposure density (500 cells ml^{-1}), larval activity (Fig. 6) was significantly lower (~50%) relative to control treatments (16%) at 16°C only ($p < 0.05$; Nemenyi post hoc test). At high cell densities (1000 and 2000 cells ml^{-1}), there were significant reductions in larval activity in the 16 and 20°C treatments relative to the control ($p < 0.05$; Nemenyi post hoc test), with all larvae being rendered immobile at these temperatures when exposed to 2000 cells ml^{-1} (Fig. 6). At warmer temperatures, decreases in activity became apparent only at the highest exposure densities (2000 cells ml^{-1}; Fig. 6), although the percentages of active larvae in these treatments (27 and 43% at 24 and 28°C, respectively) were not significantly different from control treatments ($p > 0.05$; Nemenyi post hoc test).

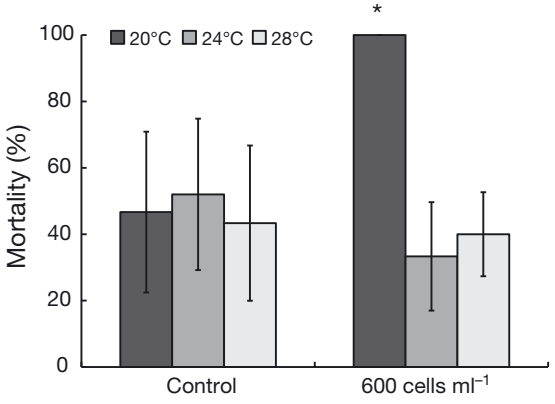

Fig. 5. Mortality of *Mercenaria mercenaria* (embryos; <4 h) exposed to 0 and 600 cells ml^{-1} of CP-1 at 20, 24, and 28°C after 24 h. Mortality at 20°C was significantly elevated relative to the control. Error bars represent ± SD; *$p < 0.001$ (Welch's *t*-test)

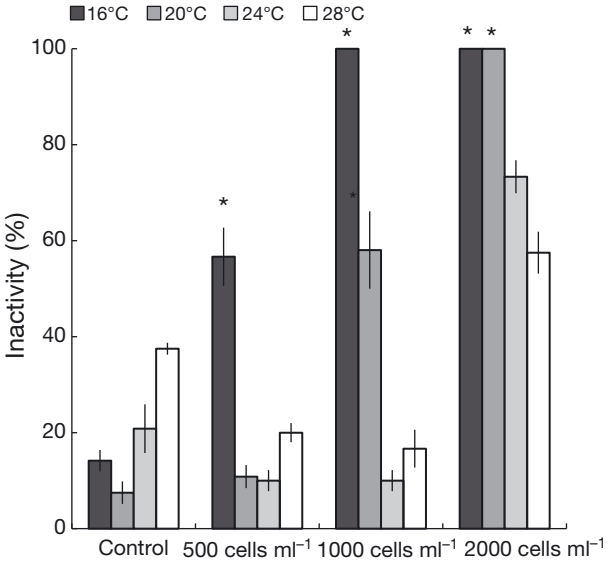

Fig. 6. Inactivity of *Argopecten irradians* (veliger; 10 d) exposed to 0, 500, 1000, and 2000 cells ml^{-1} of CP-1 at 16, 20, 24, 28°C after 24 h. Error bars represent ± SD; *$p < 0.05$ (Kruskal-Wallis test followed by a Nemenyi post hoc test, ties corrected)

Fish exposures to bloom populations

Temperature also affected the lethality of wild, bloom populations of *C. polykrikoides* to fish. Sheepshead minnows *C. variegatus* exposed to bloom water with ~5000 *C. polykrikoides* cells ml^{-1} for 24 h at 19, 23, 25, and 28°C displayed 100, 83, 17, and 17% survival, respectively (Fig. 7a), with percentage mortality being significantly greater in exposed fish at 19°C ($p < 0.05$; Marascuilo's procedure; Fig. 7a). For *M. beryllina* exposed to a bloom with ~250 cells ml^{-1}, mortalities at 19, 23, 25, and 28°C were 100, 67, 33, and 17%, respectively (Fig. 7b), with the mortality of fish exposed to *C. polykrikoides* at 19°C being significantly greater than that of fish exposed at warmer temperatures ($p < 0.05$; Marascuilo's procedure).

Chain length experiment

Chain formation by *C. polykrikoides* significantly altered its lethality to fish. The *C. polykrikoides* culture with no 4-cell chains was significantly more lethal than the culture with 35% 4-cell chains ($p <$

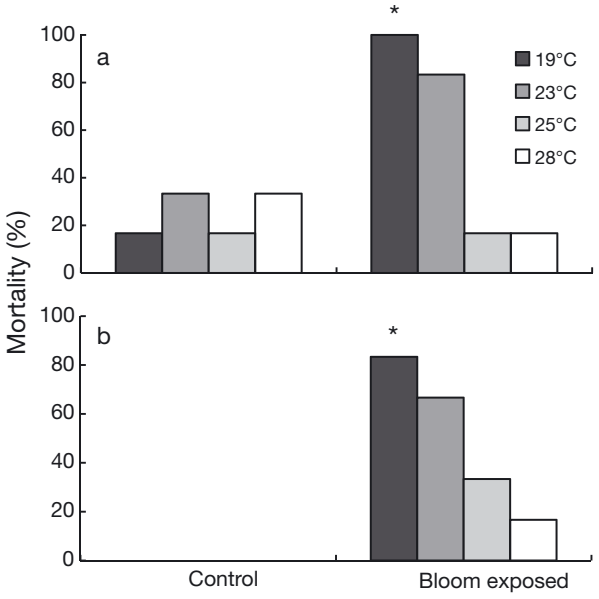

Fig. 7. Mortality of (a) *Cyprinodon variegatus* and (b) *Menidia beryllina* exposed to bloom water at 19, 23, 25, 28°C after 24 h. *p < 0.05 (Marascuilo's procedure)

0.001; Kaplan-Meier survival analysis; Fig. 8). The mean time to death of fish exposed to the culture without 4-cell chains was <1 h compared to 34 h for fish exposed to the culture comprising 35% 4-cell chains.

Catalase experiment with fish

The addition of catalase significantly mitigated the lethal effects of *C. polykrikoides* within warmer temperature treatments only ($p < 0.001$; Kaplan-Meier survival analysis; Fig. 9). All fish exposed to 2000 *C. polykrikoides* cells ml^{-1} at 28°C without catalase perished after ~1 h, whereas fish exposed to the same density of culture with the addition of catalase lived significantly longer (>4 h; $p < 0.001$; Kaplan-Meier survival analysis; Fig. 9a). Catalase had no effect on survival at cold temperature (16°C; Fig. 9b).

DISCUSSION

In repeated trials, with a variety of marine organisms, the lethality of *Cochlodinium polykrikoides* cultures and field populations was dependent upon temperature, with lower temperatures yielding greater rates of mortality and shorter times to death. High-toxicity cultures at lower temperatures had a greater abundance of single cells than less-toxic, higher-temperature cultures. Antioxidant enzymes administered at high, but not low, temperatures mitigated the lethality of *C. polykrikoides* to fish. Collectively, these findings provide new insight regarding the toxicity of this globally distributed, harmful alga and

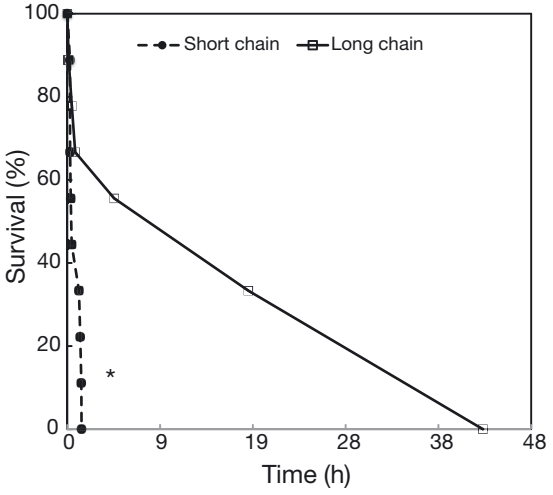

Fig. 8. Survival of *Menidia beryllina* exposed to short (4-chain = 0 ± 0%; single = 75 ± 0.5%) and long chain (4-chain = 35 ± 7%; single = 29 ± 7%) fractions of *Cochlodinium polykrikoides* at 1000 cells ml^{-1}. *p < 0.001 (Kaplan-Meier survival analysis)

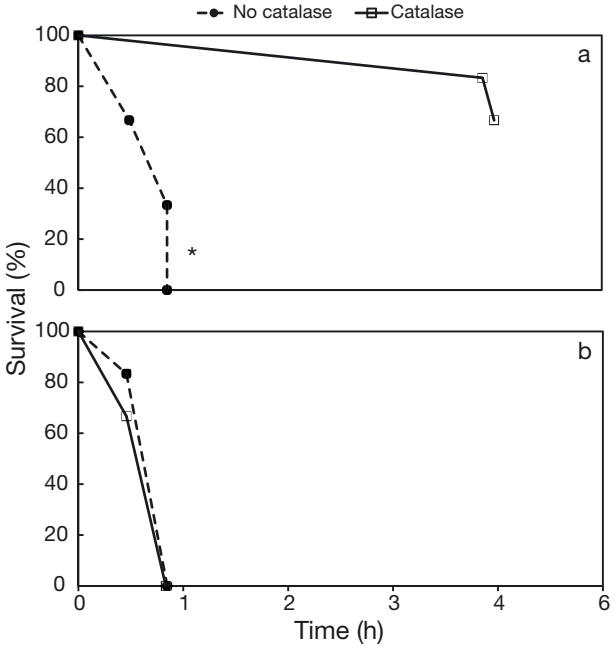

Fig. 9. Survival of *Menidia beryllina* exposed to 2000 cells ml^{-1} of *Cochlodinium polykrikoides* with and without the addition of 2.0 U ml^{-1} of catalase at (a) 28°C and (b) 16°C. The addition of catalase significantly mitigated lethal effect in only the warmer temperature treatment. *p < 0.001 (Kaplan-Meier survival analysis)

have important implications regarding the effects of HABs in marine ecosystems experiencing climate change.

Mechanisms by which temperature affects *C. polykrikoides* lethality

Prior research demonstrates that North American clones of *C. polykrikoides* cause mortality in marine organisms via the release of an extracellular compound similar to ROS. Specifically, these toxicants are reported to persist for only minutes in the absence of live cells, and can be mitigated by ROS-scavenging enzymes (e.g. catalase, peroxidase), with their potency being qualitatively proportional to their cellular growth stage (Tang & Gobler 2009a). While the precise compound causing toxicity in *C. polykrikoides* has yet to be identified, our results suggest that the toxicant is more reactive and/or stable at colder temperatures. Several common ROS compounds (e.g. OH^-, O_2^-) have fleetingly short half-lives, ranging from 10^{-6} to 10^{-9} s (Yu 1994), which are likely to be accelerated at higher temperatures due to increased scavenging and enzymatic neutralization (Wise 1995, Miller et al. 2010). Consistent with this hypothesis, we propose that the compounds released by *C. polykrikoides* are less stable at warmer temperatures, leading to milder effects on early-life-stage fish and shellfish.

Photosynthetic organisms actively produce ROS during aerobic metabolism and are reliant upon antioxidant enzymes for protection against cellular damage (Yu 1994, Apel & Hirt 2004). Chilling-enhanced photo-oxidation is a common occurrence in higher plants (Wise 1995) and occurs due to differences in stability between enzymes associated with energy-transducing (light) reactions and energy-consuming (dark) reactions at lower temperatures. With adequate light at lowered temperature, photosynthetic processes within plant tissue proceed more readily than processes involving oxygen consumption, resulting in the accumulation of ROS (Wise 1995). Consistent with this phenomenon, our experimental results demonstrate the efficacy of catalase in mitigating the toxicity of *C. polykrikoides* at higher temperatures only. We hypothesize that, at lower temperatures, similar antioxidant- and/or ROS-scavenging enzymes produced by *C. polykrikoides* and/or target organisms have a lowered ability to react with and mitigate toxicants produced by *C. polykrikoides*, leading to an enhanced mortality of fish and shellfish.

Chain formation in *C. polykrikoides* was also temperature-dependent. As growth rates increased with temperature, there was a concurrent increase in the abundance of cells in chains of 2 or more. Chain formation in red-tide-forming dinoflagellates is linked with enhanced swimming ability (Fraga et al. 1989, Selander et al. 2011), which may allow these species to remain in the photic zone during warmer periods of decreased viscosity. Temperature-dependent chain formation is also reported in the diatom *Skeletonema costatum*, with warmer temperatures yielding higher growth rates and longer chains (Takabayashi et al. 2006), potentially because rapidly growing cells divide before becoming separated. Consistent with our findings, Jiang et al. (2010) found that rapidly growing *C. polykrikoides* cultures were more likely to form chains than slower-growing cultures, and that *C. polykrikoides* blooms in NY, USA, comprised primarily 2-cell chains in early September (21°C), but were primarily single cells in late October as temperatures decreased (~15°C; Jiang et al. 2010). Similar qualitative observations regarding temperature and chain formation were made during *C. polykrikoides* blooms on the west coast of the USA (Curtiss et al. 2008, Kudela et al. 2008) and off Korea (Kim 2004).

Cultures of *C. polykrikoides* with a greater abundance of long chains were significantly less toxic than cultures dominated by single cells. This observation is consistent with studies indicating that *C. polykrikoides* produces an extracellular toxicant, such as ROS or polysaccharides (Kim et al. 1999, 2000, Kim et al. 2002), whose cellular export rates are likely dependent upon cellular surface-area-to-volume ratio. Cultures grown at higher temperatures had a greater abundance of long chains, a lower cellular surface-area-to-volume ratio, and presumably a lowered capacity to release extracellular toxicants on a per-cell basis. Accordingly, these cultures were less toxic. Collectively, these findings suggest that, while temperature may impact the physicochemical properties of toxicants and enzymes, temperature effects on chain formation are also likely to influence the lethality of *C. polykrikoides*.

Across all experiments, some variation in the toxicity of *C. polykrikoides* to fish did exist. For example, in culture experiments, clone CPSB-1G elicited more rapid times to death of *Cyprinodon variegatus* larvae than clone CP-1 at lower cell densities (see Figs. 2 & 3). Further, one collection of bloom water caused more rapid mortality in fish than another collection of bloom water. However, direct comparisons of toxicity among experiments may be problematic, due to pos-

sible variation in the sensitivities of larval fish between cohorts, as well as potential differences in the lethality of algal populations emanating from different locales. Despite any variability, across all experiments, a clear pattern emerged: populations of *C. polykrikoides* at cool temperatures were significantly more lethal compared to parallel populations held at warmer temperatures.

Implications for temperature-dependent toxicity of HABs

The lowered toxicity of *C. polykrikoides* blooms at warm temperatures has broad implications with regard to the interpretation of prior studies of this alga, bloom ecotoxicology, and future climate change. While there are conflicting reports regarding the mechanism by which *Cochlodinium* spp. cause mortality in marine life, our findings suggest that some of the prior differences in experimental findings may be related to temperature. Kim et al. (1999) measured the production of O_2^- and H_2O_2 in *C. polykrikoides* strains isolated from Korean waters and found the production of both compounds was significantly greater than in all other dinoflagellates examined (*Eutreptiella gymnastica*, *Heterosigma akashiwo*, *Prorocentrum micans*, *Gymnodinium sanguineum*, and *Alexandrium tamarense*). The authors concluded that ROS compounds likely play a large role in the lethality of *C. polykrikoides* toward marine organisms. In contrast, Kim et al. (2002) examined the production of ROS compounds by *C. polykrikoides* isolated from Japan and found the production of H_2O_2 was significantly lower than that of a known ROS-producing alga (*Chattonella marina*), leading the authors to conclude that other compounds (e.g. polysaccharides) may be responsible for the toxic effects elicited by this alga. A review of the culturing conditions used in these experiments reveals that Korean strains shown to produce large amounts of ROS (Kim et al. 1999) were maintained at 20°C, whereas Japanese strains (Kim et al. 2002) were cultured at 26°C, temperatures at which strains of *C. polykrikoides* studied here were highly lethal and more benign, respectively. While this may reflect analytical or strain-specific differences, it is plausible that the discrepancy in the assessment of ROS production by *C. polykrikoides* between Kim et al. (1999) and Kim et al. (2002) was caused by the temperature at which the 2 strains were maintained. Specifically, lower ROS production in Japanese strains may be associated with decreased ROS stability and increased reactivity with antioxidant enzymes at 26°C. There are also conflicting reports regarding the lethality of *C. polykrikoides* to fish. Dorantes-Aranda et al. (2010) suggested that *C. polykrikoides* strains isolated from Mexico (Bahía de La Paz) may be less toxic than temperate strains, as densities required to elicit lethal effects on fish were significantly greater than densities reported by studies of temperate strains (e.g. Tang & Gobler 2009b). While some strain-specific differences are likely, lower toxicity of Mexican isolates may, in part, have been due to the elevated temperatures at which they were cultured (25°C; Dorantes-Aranda et al. 2010) relative to temperate strains (21°C; Tang & Gobler 2009b).

The temperature-dependent lethality of *C. polykrikoides* described here suggests that blooms occurring in temperate latitudes may be more toxic than blooms within tropical and subtropical latitudes. Sea surface temperatures during *C. polykrikoides* blooms in tropical locales typically exceed 27°C (Anton et al. 2008, Azanza et al. 2008, Richlen et al. 2010), temperatures at which *C. polykrikoides* was significantly less toxic during the present study. During a bloom event in the Gulf of Oman, low oxygen (<1 mg l^{-1}) and mass mortality of fish communities and branching coral were reported immediately following a massive *C. polykrikoides* bloom (Marquis & Trick 2009, Bauman et al. 2010). Increased stratification, reduced solubility of oxygen, and temperature-enhanced rates of microbial respiration all likely contributed toward reduced dissolved oxygen in bloom-exposed areas. Hence, the death of marine life during the Gulf of Oman event, and perhaps other tropical *C. polykrikoides* blooms, may be the result of low dissolved oxygen rather than the production of extracellular toxicants by *C. polykrikoides*. Temperate-latitude *C. polykrikoides* blooms occur at relatively lower temperatures (14 to 25°C; Lee & Lee 2006, Gobler et al. 2008, Kudela et al. 2008, Kudela & Gobler 2012) and lethal effects, as revealed through laboratory and field investigations, are not associated with low dissolved oxygen (Gobler et al. 2008, Mulholland et al. 2009). This, in addition to current results, suggests that toxic impacts during tropical blooms may be manifested indirectly (e.g. hypoxia/anoxia), whereas lethal impacts during temperate blooms may be the result of direct contact with toxicants exuded by *C. polykrikoides*.

In temperate regions such as the northeast USA and Korea, *C. polykrikoides* blooms develop in late summer and persist into the fall (Kim et al. 2007, Tomas & Smayda 2008, Kudela & Gobler 2012).

Given declining water temperatures during this period, the results presented here suggest that as blooms progress through these seasons, their toxicity may increase. There are multiple reports of fish-killing *C. polykrikoides* blooms persisting into cooler months at temperatures below 20°C (Whyte et al. 2001, Gobler et al. 2008, Kudela & Gobler 2012), temperatures that resulted in significantly higher rates of mortality for larval fish and bivalves during this study. Because some temperate fish and shellfish species spawn in the summer and not fall (Kennedy & Krantz 1982, Bricelj et al. 1987, Kraeuter & Castagna 2001), the results presented here suggest that these early life stages may be spared the most intense effects of *C. polykrikoides* blooms. While shellfish generally exhibit greater resistance to *C. polykrikoides* than fish (Tang & Gobler 2009a), they are nonetheless susceptible, especially at the earliest life stages (i.e. embryo, trochophore, and veliger; Tang & Gobler 2009b). Some species of shellfish, including the bay scallop *Argopecten irradians*, spawn during early summer and fall months (Tettelbach et al. 2002, Tettelbach & Weinstock 2008) in regions affected by annual *C. polykrikoides* blooms (Gobler et al. 2008, Tomas & Smayda 2008), and such fall spawns may have relatively large impacts on population recruitment (Tettelbach & Weinstock 2008). The temporal overlap of *C. polykrikoides* blooms and larval *A. irradians* in fall when bloom lethality is maximal may have particularly profound effects on this population.

Sea surface temperatures are expected to increase 1 to 5°C by the year 2100 (IPCC 2014) and will likely have significant impacts on the growth, toxicity, and distribution of ichthyotoxic HAB species. Given that *C. polykrikoides* blooms on the US east coast typically occur between 14 and 25°C, but growth rates are maximal between 24 and 27°C, such warming could enhance the growth and duration of these events. Peperzak (2003, 2005) reported that a 4°C increase in sea surface temperature could yield a near doubling in growth rate for potential ROS-producing raphidophytes *Chattonella antiqua* and *Fibrocapsa japonica* (i.e. ichthyotoxic algae; Oda et al. 1997) inhabiting the North Sea. Similarly, Cloern et al. (2005) linked large-scale blooms of the ROS-producing alga *Akashiwo sanguinea* (Kim et al. 1999, Hallegraeff 2010) in San Francisco Bay to enhanced thermal stratification and warmer sea surface temperatures. Together, these observations suggest that, as sea surface temperatures continue to increase, the distribution and intensity of ichthyotoxic algal blooms may continue to expand. In areas where current sea surface temperatures are below optimum levels, increased temperatures may lead to an increase in the growth and abundance of *C. polykrikoides* and, therefore, bloom events may become more common. However, our study also suggests that *Cochlodinium* spp. blooms, at least in temperate latitudes, may become less toxic on a per-cell basis. It is possible that such temperature-controlled reductions in toxicity could be offset by temperature-induced increases in cellular growth and abundance.

Climate change is predicted to promote the expansion of HABs (Hallegraeff 2010, Hinder et al. 2012, Glibert et al. 2014). Blooms of *Cochlodinium* spp. have expanded substantially across North America, Europe, and Asia this century (Kudela & Gobler 2012). *Cochlodinium* spp. can bloom across a relatively large range of ecophysiological conditions and the continued expansion of these blooms into new, higher-latitude regions could be facilitated by increases in global ocean temperatures. The ability of *C. polykrikoides* to form resting cysts (Tang & Gobler 2012), resist zooplankton grazing (Jiang et al. 2009), allelopathically inhibit competitors (Tang & Gobler 2010), and exploit multiple forms of nutrients (Jeong et al. 2004, Gobler et al. 2012) may enable this alga, upon introduction, to become well established. Once established in new, higher-latitude, cooler regions, our findings indicate that such *C. polykrikoides* blooms would be highly toxic.

CONCLUSIONS

In conclusion, temperature strongly affected the growth and lethality of *Cochlodinium polykrikoides*. While growth increased with temperature to a maximum of 24 to 27°C, cellular toxicity decreased at warmer temperatures. As growth rates increased, the production of chains of 4 cells increased, and these longer chains were less toxic, likely due, in part, to lowered ratios of surface area to volume. Enzymatic mitigation of *C. polykrikoides* lethality was maximal at higher temperatures, suggesting that antioxidant enzymes produced by this alga and/or exposed organisms may be less protective at lower temperatures. These findings indicate that blooms developing in temperate regions during late summer are likely to be less toxic than those persisting into cooler, fall months. Additionally, *C. polykrikoides* blooms occurring at subtropical and tropical latitudes may cause mortality in marine organisms via hypoxia rather than extracellular toxicants. Finally, as global

temperatures continue to increase, *C. polykrikoides* blooms may expand into high latitudes, where they may be highly lethal.

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