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Cochlodinium polykrikoides blooms and clonal isolates from the northwest Atlantic coast cause rapid mortality in larvae of multiple bivalve species

Ying Zhong Tang · Christopher J. Gobler

Abstract Globally, many commercial bivalve populations have declined in recent decades. In addition to overharvesting and habitat loss, the increasing frequency and intensity of harmful algal blooms (HABs) are likely to contribute to bivalve losses, particularly in cases where blooms negatively impact larval stages. This paper reports on the lethal effects of clonal cultures and blooms of *Cochlodinium polykrikoides* from the US Atlantic coast on the larvae of three species of commercially and ecologically valuable bivalves: the Eastern oyster (*Crassostrea virginica*), the bay scallop (*Argopecten irradians*), and the Northern quahog (hard clam; *Mercenaria mercenaria*). Both cultures and blooms of *C. polykrikoides* were highly toxic to all three species of bivalve larvae causing 80–100% mortality during 24- to 72-h exposures at concentrations of 1–2×10^3 cells ml^{-1}. Toxicity was dependent on cell densities, growth stage of *C. polykrikoides* (i.e. cultures in exponential stage growth were more toxic than later stages), exposure time of larvae to cells (i.e. longer exposure caused higher mortality), the age of larvae (i.e. younger larvae were more sensitive), and the relative abundance of *C. polykrikoides* (i.e. the presence of other microalgae decreased toxicity). Free radical-scavenging enzymes (peroxidase and catalase) and the removal of *C. polykrikoides* cells (i.e. culture filtrate) significantly increased larval survival suggesting toxicity is maximized by contact with live cells and may involve labile toxins bound by these compounds including e.g. reactive oxygen species. The toxicity of *C. polykrikoides* to bivalve larvae was generally more severe than other HAB species (e.g. *Karenia brevis*, *Karlodinium veneficum*, *Alexandrium tamarense*, *Prorocentrum minimum*). Since the bivalves in this study spawn in the months when *C. polykrikoides* blooms on the east coast of North America, these results suggest that these blooms may have detrimental effects on efforts to restore these already diminished populations.

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Introduction

Benthic suspension feeders, such as bivalves, are important in coastal zones because they provide a variety of ecosystem services through their filtration. Many species are considered "ecosystem engineers" and have considerable commercial value (Reise 2002; Bruno et al. 2003). The filtration provided by bivalves has the potential to control eutrophication (Officer et al. 1982), increase pelagic light penetration (Newell and Koch 2004), and enrich the nutrient content of sediment (Smaal and Prins 1993) with the latter two processes providing benefit to submerged aquatic vegetation (Carroll et al. 2008; Wall et al. 2008). During the past century, many estuarine bivalve populations have suffered tremendous declines due to overharvesting, diseases, and the loss of key habitats (Jackson et al. 2001; Kemp et al. 2005; Lotze et al. 2006). Furthermore, there is growing evidence that harmful algal blooms (HABs) have negative impacts on their populations in ecosystems around the world (Jin et al. 2008) due to their synthesis of toxins and/or high levels of algal biomass (Sunda et al. 2006).

HABs afflict most temperate and tropical coastal nations, and the frequency of HAB events and their negative

impacts on fisheries have increased markedly in recent decades (Anderson et al. 2002; Hoagland et al. 2002; Heisler et al. 2008; Jin et al. 2008). HABs can be toxic to bivalves (Shumway 1990; Briceji and Shumway 1998) and more recently, the lethal effects of HABs on bivalve larvae have been documented (e.g. Matsuyama et al. 2001; Yan et al. 2001, 2003; Leverone et al. 2006; Shumway et al. 2006; Stoecker et al. 2008). Larvae represent a critical life stage for bivalve populations as reductions in the metamorphosis and survival of larvae can translate into substantial declines in adult populations (Gosselin and Qian 1997; Schneider et al. 2003; Arnold 2008). Elevated densities of *Pfiesteria piscicida* can cause rapid and complete mortality in the larvae of Eastern oysters (*Crassostrea virginica*) and bay scallops (*Argopecten irradians*; Springer et al. 2002), and *Alexandrium tamarense* can decrease the survival of the larvae of Japanese scallops (*Chlamys farreri*; Yan et al. 2001) and bay scallops (*Argopecten irradians concentricus*; Yan et al. 2003). The brown tide-forming pelagophyte *Aureococcus anophagefferens* causes reduced survival, growth, and lipid content of Northern quahog larvae (*Mercenaria mercenaria*; Padilla et al. 2006; Bricelj and MacQuarrie 2007) and can slow the growth of bay scallop larvae (Gallager et al. 1989). *Karlodinium veneficum*, an unarmoured dinoflagellate, can cause significant declines in survival of early life history stages of the Eastern oyster (Stoecker et al. 2008) and *Karenia brevis*, another unarmoured dinoflagellate, decreases survival and lengthens the development time of the larvae of Northern quahog, Eastern oyster, and bay scallop (Leverone et al. 2006).

Another dinoflagellate that may negatively impact bivalve larvae is *Cochlodinium polykrikoides*, which is notorious for causing ichthyotoxic blooms around the world. Blooms of *C. polykrikoides* reported from Japan, South Korea, the Philippines, Malaysia, Indonesia, and China (Iwataki et al. 2008) have caused hundreds of millions of dollars in fisheries losses (Kim 1998; Kim et al. 1999). In the United States, blooms of *Cochlodinium* species have become annual events on both the Atlantic and Pacific coasts (Curtiss et al. 2008; Gobler et al. 2008). While the effects of these blooms on fish are well established (Whyte et al. 2001; Gobler et al. 2008; Tang and Gobler 2009), their impacts on bivalve larvae are less clear. A field study using seawater with high abundances of *C. polykrikoides* (>10⁴ cells ml⁻¹) from the York River, Virginia, USA demonstrated that this water could rapidly kill Eastern oyster larvae (*Crassostrea virginica*; Ho and Zubkoff 1979). However, the study did not report the relative abundance of *C. polykrikoides* and other plankton in York River water and did not include experiments with clonal isolates of *C. polykrikoides* (Ho and Zubkoff 1979), making interpretation of these results difficult. In contrast, Matsuyama et al. (2001) reported that a clonal culture of

C. polykrikoides from Japan, which was concentrated by centrifugation (up to 3×10^4 cells ml⁻¹), delayed the metamorphosis of Pacific oyster larvae (*Crassostrea gigas*), but did not significantly alter their survival. Finally, Jeong et al. (2004) found that among six red tide dinoflagellates, an Asian strain of *C. polykrikoides* was an optimal prey for the larvae of the mussel *Mytilus galloprovincialis*. In addition, the difficulty in culturing *C. polykrikoides*, the variable physiological activity of *C. polykrikoides* within different growth stages (Tang and Gobler 2009), and the limited availability of robust bivalve larvae may have restricted progress to date in understanding the impacts of *C. polykrikoides* on bivalve larvae. Clearly, the impacts of *C. polykrikoides* on bivalve larvae are uncertain and to date, the impacts of North American isolates of *C. polykrikoides* on larvae have not been investigated.

The goal of this study was to assess the effects of clonal cultures of *C. polykrikoides* isolated from the US Atlantic coast and estuarine water on the survival of three species of commercially valuable and ecologically important bivalve larvae including the Eastern oyster, *Crassostrea virginica*; the bay scallop, *Argopecten irradians*; and the Northern quahog, *Mercenaria mercenaria*. We also examined the effects of co-occurring phytoplankton, of cell-removal, and of free radical-scavenging enzymes on the impact of C. *polykrikoides* on larval survival.

Materials and methods

Algal cultures

Culture isolates of *Cochlodinium polykrikoides*, strains CP1 and CPPB17, were obtained by pipetting single cells into polystyrene cell culture plates containing sterile GSe culture medium under an inverted microscope. The bloom water samples from which the cultures were established were collected on August 31, 2006 from the most western basin of the Peconic Estuary, Flanders Bay (40.923°N, 72.587°W) and September 4, 2008 from Great Peconic Bay (40.936°N, 72.512°W), New York, USA. Molecular and microscopic identification of strain CP1 has been reported previously (Gobler et al. 2008). The *C. polykrikoides* strain CPCB-10 was isolated from Cotuit Bay, Massachusetts, USA. Identification of both isolates as *C. polykrikoides* was confirmed with large subunit (LSU) rDNA sequencing (Gobler et al. 2008; Iwataki et al. 2008). Cultured *Isochrysis galbana* (Tahitian strain; T-Iso) was used as a control since it fosters maximal growth and survival in bivalve larvae (e.g. Padilla et al. 2006). All cultures were maintained in sterile GSe medium with a salinity of 31–32, made with autoclaved and 0.2 μm-filtered seawater (Doblin et al. 1999), at 21° C in an incubator with a 12 h light: 12- h dark

photoperiod, illuminated by a bank of fluorescent lights providing a light intensity of $\sim$100 µmol quanta m^{-2} s^{-1}. For all cultures, an antibiotic–antimycotic solution (a mixture of 10,000 I.U. penicillin, 10,000 µg ml^{-1} streptomycin, and 25 µg ml^{-1} amphotericin B; Mediatech. Inc., Hemdon, Virginia) was added into the medium immediately before inoculation, with a final concentration of 1% to minimize contamination by bacteria and fungus. This antibiotic mixture has no negative effects on the growth and survivorship of bivalve larvae (Padilla et al. 2006). Periodic DAPI-staining of cultures during the study indicated the general absence of bacteria in the cultures most of the time.

Bivalve larvae

Eastern oyster larvae (*Crassostrea virginica*) were provided by Dave Veilleux and Dr. Gary Wikfors of Milford laboratory, NOAA Fisheries Northeast Fisheries Science Center (Connecticut, USA); bay scallop larvae (*Argopecten irradians*) were obtained from the East Hampton Shellfish Hatchery (New York, USA), and Northern quahog larvae (*Mercenaria mercenaria*) were obtained from the Cornell Cooperative Extension shellfish hatchery facility in Southold (New York, USA) as well as from the East Hampton Town Shellfish Hatchery (New York, USA). Before experiments, all larvae were maintained in filtered seawater with daily feeding of T-Iso cultures at $\sim$5 $\times$ 10^4 cells ml^{-1} and mild aeration at $\sim$22°C.

Experiments

All experiments were conducted during June through September of 2008 with sterile, 6-well polystyrene culture plates ($n = 6$ for each treatment). For each treatment of each experiment, 10-ml experimental cultures of (CP1, CPPB17, or CPCB-10 or bloom water) of *Cochlodinium polykrikoides* were added to each well. Unless otherwise indicated, all cultures were within exponential stage growth. Approximately, 20 larvae (i.e. $\sim$2 larvae ml^{-1}) were gently transferred into each well with a modified 1.0-ml pipette, yielding a final density consistent with those used for prior experiments investigating the toxicity of harmful algae to bivalve larvae (Leverone et al. 2006; Bricelj and MacQuarrie 2007; Stoecker et al. 2008). At 24 and 72 h (except for the first scallop larva bioassay, which was 10 h), the number of dead larvae in each well was enumerated under an inverted light microscope (total magnification $\times$ 40, occasionally $\times$ 100) by scanning the entire well. The total number of larvae was determined the same way after all samples were fixed with Lugol's solution (final concentration 1%) at the end of each experiment (immediately after the final enumeration of dead larvae). A larva was considered dead when swimming and movements of the velum

and, when visible, internal organs, ceased. Experiments were conducted using 24-h-old veligers (D-stage) and with older larvae (8–11 days), which were also veligers. The single exception was an experiment conducted with 10-day-old bay scallop larvae, which were a mix of veligers and a small portion of pediveligers. All plates (with covers) were maintained with a salinity of 31–32 at light intensity of 10 µE m^{-2} s^{-1} and $\sim$24°C, a temperature yielding maximal growth rates for *Crassostrea virginica*, *Argopecten irradians*, and *Mercenaria mercenaria* larvae (Carriker 2001; Matthiessen 2001; Cragg 2006; Padilla et al. 2006).

For all experiments, a treatment of T-Iso at 1–4 $\times$ 10^4 cells ml^{-1} was used as the control, densities that yield maximal growth rates for shellfish larvae (Padilla et al. 2006). Dilution series of *C. polykrikoides* cultures (CP1, CPPB17, and CPCB-10) or bloom water were prepared by diluting treatment water with GSe medium with a salinity similar to culture and bloom water. For several experiments, a parallel series of dilutions was prepared with 1.0 to 4.0 $\times$ 10^4 cells ml^{-1} of T-Iso (identical density to the control) added into each well with the CP1 and CPCB-10 cultures. For one experiment (24-h Northern quahog larvae), 0.22 µm-filtered CP1 cultures, with and without the addition of T-Iso, were used as treatments to assess the importance of intracellular and extracellular toxicity of *C. polykrikoides*.

An experiment with Northern quahog larvae was conducted using *Cochlodinium polykrikoides* bloom water collected from Old Fort Pond, (40.868°N, 72.446°W), a tidal tributary in Eastern Shinnecock Bay, and Great Peconic Bay (40.936°N, 72.512°W), on September 5, 2008, both dominated by *C. polykrikoides*. Dilutions of bloom water were made with GSe medium as described earlier. Initial concentrations of *C. polykrikoides* and other microalgae >10 µm diameter were determined by counting cells in 1.0 ml of triplicate bloom water samples added to a Sedgewick-Rafter counting chamber after fixation with Lugol's solution. *Cochlodinium polykrikoides* abundances in the bloom water were 1.8 $\times$ 10^3 and 0.8 $\times$ 10^3 cells ml^{-1} for Shinnecock Bay (Old Fort Pond) and the Peconic Estuary (Great Peconic Bay), respectively, while the abundances of other phytoplankton >10 µm were 1.6 $\times$ 10^3 and 0.2 $\times$ 10^3 cells ml^{-1}, respectively.

Since prior research has demonstrated that reactive oxygen species (ROS)-scavenging enzymes can mitigate or reduce the toxicity of *C. polykrikoides* to fish (Kim et al. 1999; Tang and Gobler 2009), addition of peroxidase and catalase to CP1 cultures was used as treatment during experiments with bay scallop and Northern quahog larvae. The stock solutions of horseradish peroxidase (MP Biomedicals, LLC., Aurora, Ohio) and catalase (MP Biomedicals, LLC., Solon, Ohio) were added to the cultures of CP1 in a final concentration of 1.25 µg ml^{-1} for peroxidase and

Table 1 Experimental conditions for bioassays using cultures of CP1 and CPCB-10 or bloom water with *C. polykrikoides*, larvae of Eastern oyster (*Crassostrea virginica*), bay scallop (*Argopecten irradians*), and northern quahog (*Mercenaria mercenaria*)

Test	Species	Larval age (day)	Exposure times (h)	(CP1) (cells ml^{-1})	(CPCB-10) (cells ml^{-1})	(CP) in bloom water (cells ml^{-1})	(T-Iso) (cells ml^{-1})	Mean number of larvae per well ± SD (n)	(Enzymes)
1	*C. virginica*	1	24, 72	160–1,560	410–1,640	–	2.17×10^4	33 ± 15 (114)	–
2	*A. irradians*	1	10	550–2,580	–	–	4.0×10^4	7 ± 4 (48)	–
3	*A. irradians*	8	24	880–2,190	–	–	2.0×10^4	14 ± 6 (66)	–
4	*A. irradians*	10	24, 72	430–1,740	1,070–1,780	–	2.0×10^4	9 ± 3 (42)	–
5	*A. irradians*	10	24, 72	1,740	–	–	7.75×10^3	9 ± 3 (24)	1.25 μg ml^{-1} peroxidase, 0.5 U ml^{-1} catalase
6	*M. mercenaria*	1	24, 72	350–1,390	–	200–1,790	1.14×10^4	26 ± 13 (78)	–
7	*M. mercenaria*	1	72	1,300–1,410	–	–	7.95×10^4	26 ± 13 (24)	–
8	*M. mercenaria*	1	24, 72	1,390	–	–	7.95×10^4	26 ± 13 (36)	1.25 μgml^{-1} peroxidase, 0.5 U ml^{-1} catalase
9	*M. mercenaria*	11	24, 72	210–850	–	–	1.58×10^4	8 ± 3 (30)	–
10	*M. mercenaria*	1	24, 72	–	400	–	1.14×10^4	26 ± 15 (18)	–
11	*M. mercenaria*	11	24, 72	–	860	–	1.58×10^4	8 ± 5 (18)	–

Concentrations in columns 5–7 refer to ranges of series dilutions

0.5 U ml^{-1} for catalase. In the stock solutions of enzymes, horseradish peroxidase was dissolved in 10 mM phosphate buffer, 0.15 M NaCl, pH 7.4, containing 1.0% BSA and 0.1% proclin as a preservative, with a concentration of 2.5 mg ml^{-1}, while catalase was dissolved in an aqueous solution with a concentration of 1,108 U ml^{-1} (one unit decomposes 1.0 μmole of hydrogen peroxide per minute at pH 7.0, 25°C). We have previously demonstrated that these compounds at the concentrations used here do not negatively affect the growth or viability of *C. polykrikoides* (Tang and Gobler 2009).

Since the ichthyotoxicity of *C. polykrikoides* can also be affected by the growth stages of cultures (Tang and Gobler 2009), three batches of CP1 culture in exponential, stationary, and late-stationary growth phases at equal cell densities were used as treatments for a Northern quahog larvae bioassay. The cell densities of CP1, CPCB-10, and *C. polykrikoides* in bloom water, cell densities of T-Iso, species and ages of larvae, average numbers of larvae in each well, and final concentrations of peroxidase and catalase are summarized in Table 1.

Statistics

All percentage data (percent mortality) were arcsine square root-transformed before ANOVAs were performed. One-, two-, or three-way ANOVAs were performed to assess the effects of the source of *C. polykrikoides* cells (strain CP1, strain CBCP-10, or bloom water), density of *C. polykrikoides* cells, the duration of larval exposure to *C. polykrikoides* cells (24 or 72 h), the addition of other microalgae (T-Iso), the addition of enzymes, and/or culture filtrate on larval

survival. Only subsets of these factors were part of each experiment. Differences among treatments were generally assessed with post hoc Holm-Sidak methods for multiple pairwise comparisons with SigmaStat 3.1. When transformations of non-normally distributed data sets were unsuccessful, a Kruskal–Wallis ANOVA on ranks was employed. In all cases, significance levels were set at $P < 0.05$.

Results

Toxicity of *C. polykrikoides* to larvae of the Eastern oyster (*Crassostrea virginica*)

Both strains of *C. polykrikoides*, CP1 and CPCB-10, caused dramatic mortality of 24-h *C. virginica* larvae (Fig. 1). In a 24-h exposure, the culture of CP1 (1.56×10^3 cells ml^{-1}) and CPCB-10 (1.64×10^3 cells ml^{-1}) caused 94 and 64% mortality compared to complete survival in control treatments, while lower cell densities also caused elevated (i.e. significantly higher than in the control) mortalities in oyster larvae (Fig. 1). After 72 h of exposure, CPCB-10 caused higher mortality than did the 24-h exposure for all cell densities, as did CP1 at lower cell densities only (0.39–0.78×10^3 cells ml^{-1}; Fig. 1). However, although cell density of *C. polykrikoides* had a statistically significant treatment effect on oyster larvae survival (Three-way ANOVA, $P < 0.001$), strains and exposure time did not (Three-way ANOVA, $P > 0.05$). Even the lowest *C. polykrikoides* densities (0.16×10^3 cells ml^{-1}) yielded mortality in oyster larvae significantly higher than that in the control
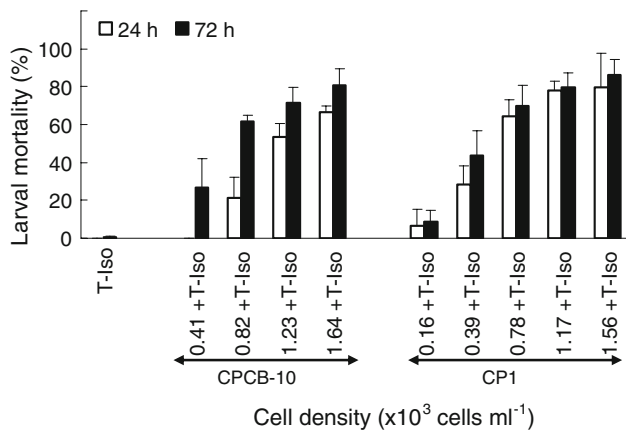
Fig. 1 Eastern oyster (*Crassostrea virginica*) larval bioassay for *C. polykrikoides* strains CP1 and CPCB-10, showing relationship between mortality of larvae and cell density of CP1 and CPCB-10. Larvae were 4-day-old, and the final concentration of *Isochrysis galbana* (T-Iso) in negative control and treatments was 2.2×10^4 cells ml^{-1}. *Error bars* indicate SD of 6 replicates

(Holm-Sidak post hoc pairwise comparison (termed post hoc comparison onward); $P < 0.001$; Fig. 1).
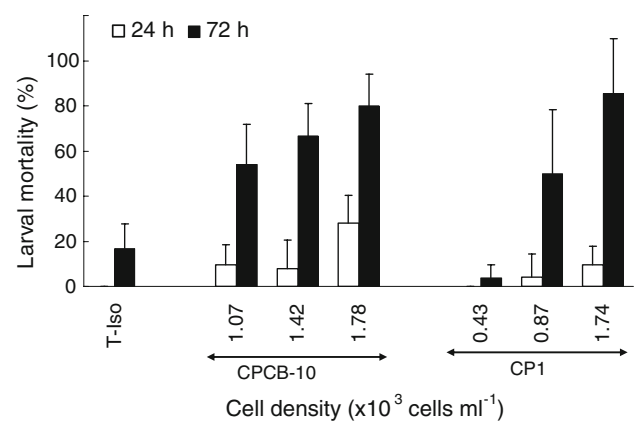
Toxicity of *C. polykrikoides* cultures to larvae of bay scallops (*Argopecten irradians*)

Cochlodinium polykrikoides (CP1, 2.58×10^3 cells ml^{-1}) caused 99% mortality in 24-h scallop larvae after a 10-h exposure compared to 8.5% in the control (Fig. 2a). Lower densities of CP1 yielded lower larval mortalities, which were still significantly higher than that of the control (post hoc comparison; $P < 0.001$; Fig 2a). Eight-day-old larvae exposed to CP1 for 24 h experienced mortalities of 60 to 100% at cell densities from 0.88 to 2.19×10^3 cells ml^{-1}, all of which were significantly higher than mortalities in the control (post hoc comparison; Fig. 2b). The addition of T-Iso to CP1 significantly lessened its toxic effect on 8-days larvae at cell densities $>0.88 \times 10^3$ cells ml^{-1} ($P < 0.001$, two-way ANOVA; Fig. 2b).

Ten-day larvae were more resistant to *C. polykrikoides* cultures than the younger larvae (Fig. 3). For example, 10-days larvae exposed to 1.7×10^3 CP1 cells ml^{-1} displayed only 9.6% mortality in 24 h (Fig. 3), whereas 24-h larvae exposed to 1.5×10^3 CP1 cells ml^{-1} for 10 h experienced 83% mortality (Fig. 1a). In general, the mortality of 10-days larvae was dependent on *C. polykrikoides* cell densities ($P < 0.001$; Three-way ANOVA) but did not differ between strains CP1 and CB-CP10 ($P > 0.05$; Three-way ANOVA). For example, strain CPCB-10 caused larval mortalities of 54–80% at cell densities of 1.07 to 1.78×10^3 cells ml^{-1} in 72 h, while CP1 caused similar mortalities (50 and 89% at cell densities of 0.87 and 1.74×10^3 cells ml^{-1}; Fig. 3). Finally, exposure time had a
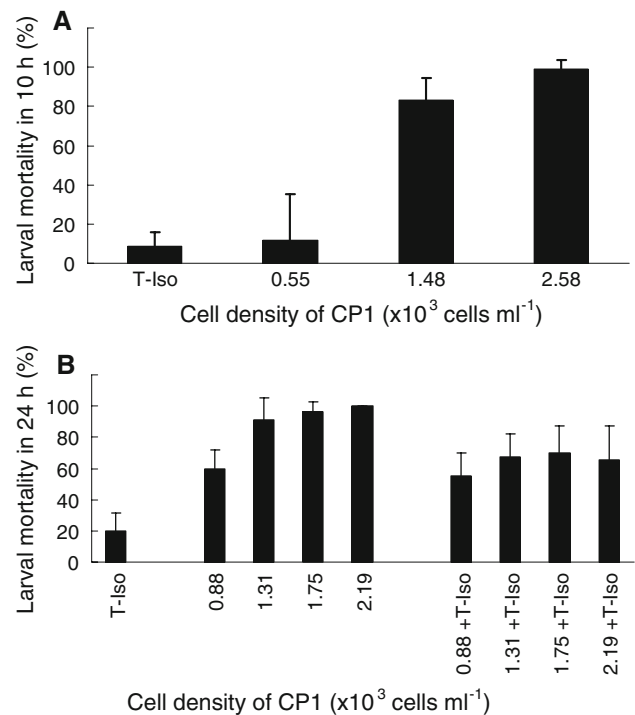
Fig. 2 Bay scallop (*Argopecten irradians*) larval bioassays for *C. polykrikoides* CP1, showing relationship between mortality of larvae and cell density of CP1 and mitigation effect of *Isochrysis galbana* (T-Iso) on toxicity. **a** Experiment using larvae of 24 h old. Exposure time of larvae to CP1 culture was 10 h, and cell density of T-Iso control was 4.0×10^4 cells ml^{-1}; **b** experiment using larvae of 8-day-old and using CP1 culture with and without addition of T-Iso. Exposure time was 24 h. Cell density of T-Iso in control and CP1 culture was 2.2×10^4 cells ml^{-1}. *Error bars* indicate SD of 6 replicates

Fig. 3 Bay scallop (*Argopecten irradians*) larval bioassays for *C. polykrikoides* strains CP1 and CPCB-10, showing toxicity of CP1 and CPCB-10 to 10-day-old larvae in 24 and 72 h. Cell density of T-Iso control was 7.8×10^3 cells ml^{-1}. *Error bars* indicate SD of 6 replicates

significant treatment effect, as 72-h exposure yielded three- to ten-times greater mortalities than those observed at 24 h at densities $\geq 8.7 \times 10^2$ cells ml^{-1} ($P < 0.05$; Three-way ANOVA; post hoc comparison).

Toxicity of *C. polykrikoides* to the larvae of Northern quahog (*Mercenaria mercenaria*)

One-day-old Northern quahog larvae were exposed to cultures of T-Iso (control), CP1 (with equal level of T-Iso), and *C. polykrikoides* bloom water from Peconic Bay (PB) and Old Fort Pond (OFP) for 24 h and 72 h (Fig. 4a). The CP1 culture caused mortalities in larvae from 9 to 59% in 24 h and 14 to 84% at 72 h at cell densities from 0.35 to 1.39×10^3 cells ml^{-1} (Fig. 4a). Bloom water from PB (*C. polykrikoides* cell density 0.79×10^3 ml^{-1}) caused larval mortalities of 5–23% in 24 h and 27–97% in a 72-h exposure with different dilutions (Fig. 4a), while bloom water from OFP (*C. polykrikoides* cell density 1.79×10^3 ml^{-1}) was less potent, causing mortalities of only 11% in 24 h and 23% in 72 h (Fig. 4a). Statistically, *C. polykrikoides* cell densities, the source of *C. polykrikoides* cells (CP1 culture vs. PB vs. OFP), and exposure time (24 vs. 72 h) all had significant treatment effects (Three-way ANOVA; $P < 0.0001$ for each; Fig. 4a): mortality of larvae during the 72-h exposure was significantly greater than those exposed for 24 h (post hoc comparison; $P < 0.001$; Fig. 4a); mortality of larvae exposed to PB bloom water was significantly greater than both other sources of *C. polykrikoides* cells (post hoc comparison; $P < 0.001$; Fig. 4a), while mortality of larvae exposed to CP1 was significantly greater than that of OFP bloom water (post hoc comparison; $P < 0.001$; Fig. 4a). Finally, the mortality of larvae exposed to even the lowest doses of *C. polykrikoides* cells was significantly greater than those in the T-Iso control (post hoc comparison; $P < 0.001$; Fig. 4a).

Eleven-day-old Northern quahog larvae were also quite sensitive to *C. polykrikoides* (Fig. 4b). After a 24-h exposure, CP1 cultures at cell densities of 0.21 to 0.85×10^3 cells ml^{-1} caused larval mortalities from 31 to 71% (Fig. 4b). After 72 h of exposure, the mortalities increased but not significantly (Two-way ANOVA; $P > 0.05$; Fig. 4b). For this experiment, the density of *C. polykrikoides* cells had a significant treatment effect (Two-way ANOVA; $P < 0.001$). All densities of *C. polykrikoides* $>0.42 \times 10^3$ cells ml^{-1} yielded larval mortality exceeding the control (post hoc comparison; $P < 0.001$; Fig. 4b).

Cochlodinium polykrikoides strain CPCB-10 was also highly toxic to Northern quahog larvae. For example, 0.40×10^3 *C. polykrikoides* cells ml^{-1} killed 17% of 24-h larvae in 24 h and 20% in 72 h, while the corresponding mortalities of larvae exposed to the CPCB-10 culture with addition of T-Iso were 11% in 24 h and 24% in 72 h (Table 2). In contrast, control treatments displayed complete survival during this experiment (Table 2). While the addition of T-Iso to cultures did not alter the survival of larvae exposed to CPCB-10, mortalities of the larvae were significantly higher in both treatments with CPCB-10
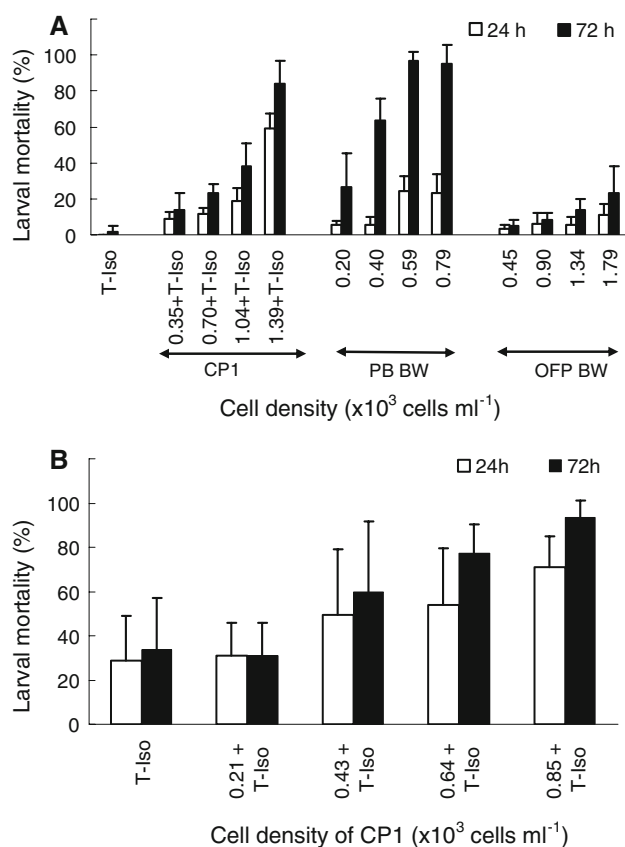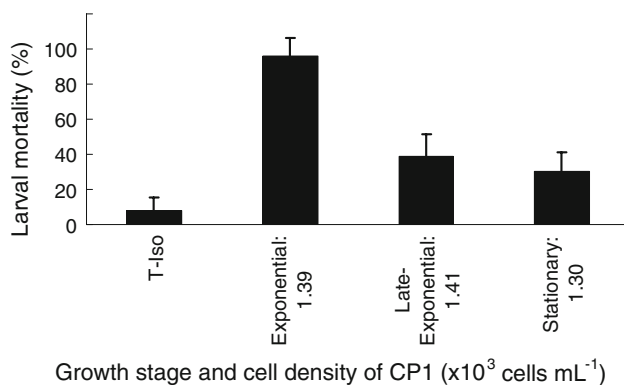

Fig. 4 Northern quahog (*Mercenaria mercenaria*) larval bioassays for *C. polykrikoides* strain CP1 and *C. polykrikoides* bloom water collected from Peconic Bay (PB BW) and Old Fort Pond (OFP BW), New York, on September 5, 2008, showing relationship between larval mortality and cell density of *C. polykrikoides*. **a** Experiment using 24-h-old larvae exposed to CP1 culture (with addition of T-Iso) and bloom water. Exposure time of larvae to cultures and bloom water was 72 h, and cell density of T-Iso in control and CP1 culture was 1.1×10^4 cells ml^{-1}; **b** experiment using larvae of 11-day-old and CP1 culture with addition of T-Iso. Exposure time was 72 h and cell density of T-Iso in control and CP1 culture was 1.6×10^4 cells ml^{-1}. *Error bars* indicate SD of 6 replicates

compared to the control (post hoc comparison; $P < 0.001$; Table 2), and mortalities were significantly higher after 72 h compared to the 24-h exposure (post hoc comparison; $P < 0.001$; Table 2). Older larvae (11 days) were similarly susceptible to CPCB-10 as cell densities of 0.86×10^3 cells ml^{-1} caused 59 and 54% mortalities without and with the addition of T-Iso after a 24-h exposure and significantly higher mortalities (97 and 80%) after the 72-h exposure (post hoc comparison; $P < 0.001$; Table 2). The mortality of the 11-days larvae was significantly higher in all treatments with *C. polykrikoides* CPCB-10 compared to the control (post hoc comparison; $P < 0.001$; Table 2). Finally, *C. polykrikoides* strain CPPB17 caused significantly greater mortality in 24-h Northern quahog larvae compared to control treatments (post hoc comparison; $P < 0.001$; Table 2).

Table 2 Effect of cultures of *C. polykrikoides* CPCB-10 and CPPB-17, with or without addition of *Isochrysis* (T-Iso), on survival of larval Northern quahog (*Mercenaria mercenaria*) of different ages

Larval age (day)	Cell density of *C. polykrikoides* (cells ml^{-1})	Cell density of T-Iso (cells ml^{-1})	Larval mortality (%) in 24 h (mean $\pm$ 1 SD, $n = 6$)	Larval mortality (%) in 72 h (mean $\pm$ 1 SD, $n = 6$)
1	0	11×10^3 (control)	0 ± 0	2 ± 4
1	CPCB-10: 0.4×10^3	0	17 ± 6	20 ± 6
1	CPCB-10: 0.4×10^3	11×10^3	11 ± 2	24 ± 18
11	0	16×10^3 (control)	29 ± 20	34 ± 23
11	CPCB-10: 0.86×10^3	0	59 ± 27	97 ± 7
11	CPCB-10: 0.86×10^3	16×10^3	54 ± 35	79 ± 20
1	0	1.65×10^4 (control)	0	0
1	CPPB-17: 350	16×10^4	13 ± 5	27 ± 14
1	CPPB-17: 350	0	7.5 ± 4	35 ± 12

Exposure time of larvae to cultures was 24 and 72 h for all three experiments

Fig. 5 Northern quahog (*Mercenaria mercenaria*) larval bioassays for *C. polykrikoides* strain CP1, showing effect of growth stage on toxicity of CP1 culture to 24-h-old larvae. Exposure time of larvae to cultures was 72 h, and cell density of T-Iso control was 7.95×10^3 cells ml^{-1}. *Error bars* indicate SD of 6 replicates

Effects of growth stage and enzymes on toxicity of *C. polykrikoides* to bivalve larvae

Cochlodinium polykrikoides cultures in exponential growth were significantly more toxic to the 24-h Northern quahog larvae after a 72-h exposure (96% mortality) than late exponential (39% mortality) and stationary stages (31% mortality; post hoc comparison; $P < 0.001$; Fig. 5), while the later two showed similar toxicity (post hoc comparison; $P > 0.05$). Additions of the enzymes peroxidase (final concentration 1.25 µg ml^{-1}) and catalase (final concentration 0.5 U ml^{-1}) to the culture of CP1 with a cell density of 1.74×10^3 cells ml^{-1} significantly reduced the mortality of 10-day bay scallop larvae from 85 to 27 and 61%, respectively, during a 72-h exposure (post hoc comparison, $P < 0.001$; Fig. 6a). The addition of peroxidase was more effective in mitigating the toxicity of CP1 to scallop larvae than catalase (post hoc comparison; $P < 0.05$). Peroxidase

(1.25 µg ml^{-1}) and catalase (0.5 U ml^{-1}) also significantly reduced the mortality of 24-h Northern quahog larvae in 24 and 72-h exposures to CP1 (Two-way ANOVA, post hoc comparison, $P < 0.001$; Fig. 6b), with a more significant mitigation effect observed during the 24-h exposure compared to the 72-h exposure (Two-way ANOVA, post hoc comparison, $P < 0.001$; Fig. 6b). Again, the addition of peroxidase was more effective than catalase in mitigating the toxicity in a 24-h exposure (post hoc comparison, $P < 0.01$; Fig. 6b). The mortality of Northern quahog larvae in the filtrate of CP1 culture (3 and 6% mortalities in 24 and 72-h exposures, respectively) was nearly identical to that in the T-Iso control (4 and 8% mortalities in 24 and 72-h exposures, respectively; ANOVA post hoc comparison, $P > 0.9$) and was significantly lower than for the exposure to the whole culture (81 and 96% mortalities in 24 and 72-h exposures, respectively, post hoc comparison, $P < 0.001$; Fig. 6b).

Discussion

This study has explicitly demonstrated the highly toxic activity of *Cochlodinium polykrikoides* cultures and bloom water from the northeast coast of North America to larvae of three bivalve species: the Eastern oyster, the bay scallop, and the Northern quahog (*Crassostrea virginica*, *Argopecten irradians*, and *Mercenaria mercenaria*). The toxicity of *C. polykrikoides* cultures (CP1, CPCB-10, and CPPB17) and bloom water (Great Peconic Bay, Shinnecock Bay, New York, USA) to bivalve larvae was dependent on cell densities (both in cultures and in bloom water), growth stage of *C. polykrikoides* (i.e. cultures in exponential growth were more toxic than that at later stages), exposure time of larvae to cells (i.e. longer exposure caused higher mortality), the age of larvae (i.e. younger larvae were more susceptible), and the relative abundance of *C. polykrikoides*
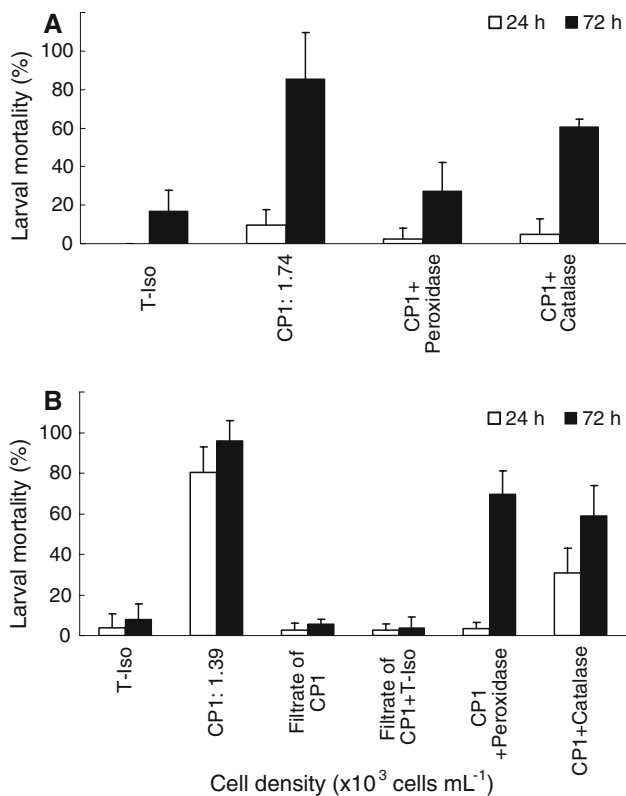
Fig. 6 a Bay scallop (*Argopecten irradians*) (10-day-old) and **b** Northern quahog (*Mercenaria mercenaria*) (24 h old) larval bioassays for *C. polykrikoides* strain CP1, showing effect of addition of peroxidase (final concentration 1.25 µg ml^{-1}) and catalase (final concentration 0.5 U ml^{-1}) to the whole culture on toxicity. The T-Iso culture (7.75×10^3 and 7.95×10^3 cells ml^{-1} for scallop and clam, respectively) and, for clam larvae only, the filtrate of CP1 culture through 0.22-µm mesh (with and without T-Iso) were used as negative controls, while the initial cultures of CP1 were used as positive controls. *Error bars* indicate SD of 6 replicates

(i.e. the presence of other microalgae decreased toxicity). The dependence of toxicity on the cell density, growth stage, exposure time, age of larvae, and presence of other microalgae is consistent with previous investigations, which have characterized the toxicity of this species to finfish (Tang and Gobler 2009). The greater toxicity of exponential stage cultures and cultures with higher cell abundances described here is also consistent with the findings of Kim et al. (1999) who investigated Asian strains of *C. polykrikoides*. The greater resistance of older larvae of scallops and Northern quahog to the toxicity of *C. polykrikoides* may be due to their more calcified shells (Carriker 2001) and/or ingestion of *C. polykrikoides* cells during incubations (thus reducing the density of toxic cells; Jeong et al. 2004). However, this finding differs from the toxic effect of *Karlodinium veneficum* to Eastern oyster larvae, which were more sensitive at later stages (Stoecker et al. 2008). Since older larvae consume more prey, the differences in toxicity between *C. polykrikoides* and *K. veneficum*

to older larvae suggests that *K. veneficum* toxicity involves an intracellular toxin which is consumed in higher quantities by older individuals (i.e. karlotoxin; Stoecker et al. 2008), whereas *C. polykrikoides* toxicity does not. The significant reduction in mortalities of bay scallop and Northern quahog larvae by the removal of *C. polykrikoides* cells (i.e. culture filtrate) or the addition of the enzymes peroxidase and catalase to a CP1 culture was also consistent with previous investigations of juvenile finfish (Tang and Gobler 2009). These findings suggest that a similar toxic mechanism is responsible for killing finfish and bivalve larvae and that toxicity is maximized by close contact between viable cells and victim organisms and may involve highly labile toxins, such as reactive oxygen species which can be scavenged by peroxidase and catalase (Kim et al. 1999; Tang and Gobler 2009). However, further study is warranted to establish the mortality mechanism associated with *C. polykrikoides*.

Previously, a field study using seawater containing high abundances of *C. polykrikoides* cells ($>10^4$ cells ml^{-1}) demonstrated this bloom water could rapidly kill Eastern oyster larvae (*Crassostrea virginica*; Ho and Zubkoff 1979). However, the levels of mortality of oyster larvae ($\sim$90%) exposed to 10^4 cells ml^{-1} were similar to our observations of cultures and some blooms at order of magnitude lower densities. Ho and Zubkoff (1979) suggested that spatial competition was the cause of adverse effects that the bloom water posed to oyster larvae. However, this hypothesis would not account for the mitigation effect of additional microalgae or scavenging enzymes (peroxidase and catalase) on the toxicity of C. *polykrikoides* cultures, suggesting other factors, such as reactive toxins, contribute toward larval mortality.

Although we did not detect differences in toxicity among different clonal isolates of *C. polykrikoides* to bivalve larvae, there were differences among different growth stages of cultures and between blooms from different locations. For example, bloom water from the Peconic Estuary with 0.8×10^3 cells ml^{-1} of *C. polykrikoides* was significantly more lethal to Northern quahog larvae than bloom water from Old Fort Pond with more than twice the *C. polykrikoides* density (1.8×10^3 cells ml^{-1}). These differences may be a function of the physiological activity of cells at different growth stages, or mitigation by the presence of other plankton, or both factors. The presence of other microalgae can significantly reduce the toxic effects of *C. polykrikoides* cultures to finfish (Tang and Gobler 2009) and larvae (present study). In the Peconic Estuary bloom water, there were eight-times fewer non-*C. polykrikoides* microalgae (0.2×10^3 cells ml^{-1}) present compared to Old Fort Pond (1.6×10^3 cells ml^{-1}), suggesting that other microalgae were more likely to mitigate the toxicity of the Old Fort Pond bloom. Furthermore, the lower toxicity of the Old

Table 3 Comparison of toxicity of *C. polykrikoides* and other harmful dinoflagellate species to bivalve larvae

HAB species	Concentration (cells ml^{-1})	Larvae species	Larval age (day)	Exposure time (day)	Mortality (%)	References
C. polykrikoides	1–2×10^3	*C. virginica* *A. irradians* *M. mercenaria*	1–10	1–3	80–100	This study
Alexandrium tamarense	1×10^4	*Chlamys farreri* *A. irradians concentricus*	–	3	<10	Yan et al. (2001, 2003)
Prorocentrum minimum	1×10^4	*C. virginica*	–		0	Stoecker et al. (2008)
Prorocentrum minimum	1×10^4	*C. virginica*	–	>10	Minimal	Wikfors and Smolowitz (1995)
Karlodinium veneficum	1×10^4	*C. virginica*	1	2	<15%	Stoecker et al. (2008)
Karlodinium veneficum	1×10^6	*C. virginica*	1	3	∼90	Stoecker et al. (2008)
Pfiesteria shumwayae	$>3 \times 10^3$	*C. virginica*		1	∼60	Shumway et al. (2006)
Karenia brevis	5×10^3	*C. virginica*	1–3	3	81	Leverone et al. (2006)
Karenia brevis	5×10^3	*A. irradians*	1–3	3	63	Leverone et al. (2006)
Karenia brevis	5×10^3	*M. mercenaria*	1–3	3	74	Leverone et al. (2006)
Pfiesteria piscicida	5×10^3	*A. irradians*	–	0.04	100	Springer et al. (2002)

Karlodinium veneficum experiments used the strain CCMP1974, and *Karenia brevis* experiments used the Wilson clone

Fort Pond bloom might also reflect a lower growth rate (or late growth stage) of that population since the cell densities found there (1.8×10^3 cells ml^{-1}) were close to the maximum obtained in culture and thus may have been in a later, and thus less toxic, stage of growth. Altered physiological activity of cells may also account for the low toxicity of Asian *C. polykrikoides* cultures to larval Pacific oysters (*Crassostrea gigas*) reported by Matsuyama et al. (2001), where a cell density of 3.0×10^4 cells ml^{-1} caused only 5% mortality. Although the cell abundances used by Matsuyama et al. (2001) were ∼10-times higher than those used in the present study, they were achieved by centrifugation, a process that causes this species to lose viability and toxicity (Tang and Gobler 2009). Since the toxicity of *C. polykrikoides* can vary widely depending on the viability and physiological status of cultures (Fig 5; Tang and Gobler 2009), cell dosage and median lethal concentration (LC50) concept are not ideal indices of of *C. polykrikoides* toxicity.

The toxic effects of *C. polykrikoides* on bivalve larvae seem to be more severe than most harmful/toxic dinoflagellates such as *Alexandrium tamarense* (Yan et al. 2001, 2003), *Prorocentrum minimum* (Wikfors and Smolowitz 1995; Stoecker et al. 2008), *Karlodinium veneficum* (Stoecker et al. 2008), *Pfiesteria shumwayae* (Shumway et al. 2006), and *Karenia brevis* (Leverone et al. 2006), as compared in Table 3 on a per cell basis. The most toxic strains of *Pfiesteria piscicida*, however, caused 100% mortality in larval bay scallops (*A. irradians*) at cell densities of 5×10^3 cells ml^{-1} after only 1-h exposure (Springer et al. 2002), indicating *P. piscicida* is more lethal to scallop larvae than *C. polykrikoides*.

The cell abundances of *C. polykrikoides* used in our experiments ($\leq 2.2 \times 10^3$ cells ml^{-1}) were equivalent to levels found in blooms in US estuaries (Gobler et al. 2008). Since the bivalve species used in our study spawn in months (June to October; Barber and Blake 1983; Hesselman et al. 1989; Thompson et al. 1996; Tettelbach et al. 2002) overlapping with the occurrence of *C. polykrikoides* blooms on the east coast of North America (July to October; Nuzzi 2004; Gobler et al. 2008), our results suggest that blooms of *C. polykrikoides* may have severe negative impacts on Eastern oyster, bay scallop, and Northern quahog populations. Since many wild populations of bivalves, including the species studied here, have experienced precipitous declines (Jackson et al. 2001; Kemp et al. 2005; Lotze et al. 2006; Myers et al. 2007), future management and restoration planning should consider the temporal and spatial range of *C. polykrikoides* and other harmful algal blooms.

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