Allelopathic effects of *Cochlodinium polykrikoides* isolates and blooms from the estuaries of Long Island, New York, on co-occurring phytoplankton

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ABSTRACT: The toxic dinoflagellate *Cochlodinium polykrikoides* forms harmful algal blooms in coastal ecosystems around the world, to the great detriment of fisheries. Here we describe the allelopathic effects of *C. polykrikoides* blooms and strains isolated from east coast estuaries of the United States on natural communities and cultured phytoplankton. The allelopathic effects of *C. polykrikoides* cultures and blooms on the target microalgal cells included loss of motility, distortion of cell morphology, and 60 to 100 % cell mortality within minutes to 24 h. Allelopathy by *C. polykrikoides* on target microalgae was dependent on the relative and absolute cell abundance of each species as well as exposure time. The ability of *C. polykrikoides* cultures to kill target algae connected through a 5 μm mesh nylon membrane indicated that the allelopathic agents were extracellular and that direct cellular contact with target cells was not required for *C. polykrikoides* to exhibit allelopathy. Freezing, heating, sonication, and filtration of *C. polykrikoides* cells led to the complete loss of their allelopathic effect, suggesting that the allelopathic agents were short-lived and dependent on cell viability. Additions of the reactive oxygen species (ROS)-scavenging enzymes peroxidase and catalase into *C. polykrikoides* cultures eliminated or lessened their allelopathic effects, suggesting ROS-like chemical(s) were responsible for the allelopathy. These results are consistent with bioassays of fish and shellfish larvae with *C. polykrikoides*, suggesting that its toxicity to fish and shellfish larvae and its allelopathic effects on algae are caused by the same agents. The rapid and strong allelopathic effects exhibited by *C. polykrikoides* suggest allelopathy may facilitate blooms by eliminating competing species.

KEY WORDS: Allelopathy · *Cochlodinium polykrikoides* · Harmful algal blooms · Reactive oxygen species · Toxicity · Physiology · Ecology · Phytoplankton

INTRODUCTION

Harmful algal blooms (HABs) represent a serious threat to the inhabitants of coastal ecosystems across the globe as they negatively impact marine life, habitats, and economies. Anthropogenic nutrient loading and physical oceanographic processes are known to play a central role in the outbreak of HABs (Paerl 1988, Hallegraeff 1993, Pitcher & Calder 2000, McGillicuddy et al. 2005, Anderson et al. 2008, Heisler et al. 2008). Biological interactions among the plankton are also important for the development and sustenance of HABs. Among these, the ability or inability of pelagic (Turner & Tester 1997, Gobler et al. 2004, Buskey 2008) and benthic grazers (Bricelj & Shumway 1998, Cerrato et al. 2004, Hegaret et al. 2007) to consume bloom-forming microalgae has been most commonly investigated. Another biological factor, which may strongly influence the dynamics of HABs, but has been less frequently considered, is allelopathy.

Allelopathy has long been known to play a crucial role in the ecology of terrestrial plants (Muller 1966, Putnam & Duke 1974, Rice 1984, Siqueria et al. 1991). Phytoplankton showing allelopathic effects on other algae, bacteria, and grazers include both freshwater and marine species, particularly diatoms, cyanobacte-

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ria, haptophytes, and dinoflagellates (Legrand et al. 2003, Granéli & Hansen 2006, Tillmann et al. 2008b). More recently, several phytoplankton species forming HABs have been found to have allelopathic effects on other microalgae (Legrand et al. 2003, Granéli & Hansen 2006, Tillmann et al. 2008b). Allelopathy has been purported to regulate both phytoplankton community composition and HAB dynamics (Legrand et al. 2003, Granéli & Hansen 2006, Tillmann et al. 2008b), although, in many cases, the responsible allelochemical agents have not been identified (Legrand et al. 2003, Granéli & Hansen 2006, Tillmann et al. 2008b). Others suggest allelopathy may be important for HAB maintenance, but not for bloom initiation (e.g. Solé et al. 2006, Jonsson et al. 2009). The allelopathic effects of HABs are often caused by extracellular chemical agents that are different from the chemicals responsible for poisoning humans via shellfish (Tillmann & John 2002, Legrand et al. 2003, Granéli & Hansen 2006, Prince et al. 2008, Tillmann et al. 2008b).

Another dinoflagellate that may have allelopathic impacts on microalgae is *Cochlodinium polykrikoides*. *C. polykrikoides* has caused ichthyotoxic blooms around the world. In Asia, blooms of *C. polykrikoides* have been reported in Japan (Iwataki et al. 2008), South Korea (Kim 1998, Kim et al. 1999), the Philippines (Azanza et al. 2008), Malaysia (Anton et al. 2008), Indonesia (Anton et al. 2008), the Arabian Gulf (Richlen et al. 2010), and China (Qi et al. 1993) and have caused hundreds of millions of dollars in fisheries losses (Kim 1998, Kim et al. 1999). In North America, blooms of at least 2 species of *Cochlodinium*, including *C. polykrikoides*, have occurred in the United States (Silva 1967, Marshall 1995, Kudela et al. 2008, Tomas & Smayda 2008), Canada (Whyte et al. 2001), the Caribbean (Margalef 1961), Costa Rica (Guzmán et al. 1990), and Mexico (Gárate-Lizárraga et al. 2004) and have occurred annually since 2004 on both the Pacific and Atlantic coasts (Curtiss et al. 2008, Gobler et al. 2008, Mulholland et al. 2009). The highly lethal effects of these blooms on fish, shellfish, shellfish larvae, and zooplankton and subsequent impacts on fisheries have been well established (Kim et al. 1999, Whyte et al. 2001, Gobler et al. 2008, Jiang et al. 2009, Tang & Gobler 2009a, b). Studies to date suggest short-lived, labile toxins, similar to reactive oxygen species (ROS), play a central role in the toxicity of *C. polykrikoides* to fish and shellfish (adult, juvenile, and larvae) (Kim et al. 1999, Jiang et al. 2009, Tang & Gobler 2009a, b). Both this nature of toxicity and the near-monospecific nature of *C. polykrikoides* bloom patches suggest this species may also have allelopathic effects on competing microalgae. Many harmful algae that are ichthyotoxic are also known to have allelopathic impacts on other algae, including *Karenia brevis* (Kubanek et al. 2005, Prince et al. 2008),

Prymnesium parvum (Fistarol et al. 2003, Granéli & Johansson 2003, Tillmann 2003), *Alexandrium* spp. (Arzul et al. 1999, Tillmann & John 2002, Fistarol et al. 2004), *Chrysochromulina polylepis* (Schmidt & Hansen 2001), and *Heterosigma akashiwo* (Yamasaki et al. 2009). However, to date, the allelopathic effects of isolates of *Cochlodinium* species on other microalgae have not been documented. Yamasaki et al. (2007) observed growth inhibition and formation of abnormal cells in *Akashiwo sanguinea* co-cultured with *C. polykrikoides*, but allelopathy was clearly excluded from their explanations of the possible causes, since a low-cell-density culture of *C. polykrikoides* connected with a culture of *A. sanguinea* through a 3 µm mesh membrane did not exhibit the inhibitory effect. Instead, the observed growth inhibition was interpreted with a mechanism requiring direct cell contact.

Here we present our investigation on the allelopathic impacts of *Cochlodinium polykrikoides* clones and blooms from Long Island, New York, USA, to various species of phytoplankton (target species) that are indigenous to most coastal waters. Cultures of the target species exposed to or co-cultured with *C. polykrikoides* and connected to *C. polykrikoides* cultures through a 5 µm mesh exhibited dramatic mortalities and declines in cell densities during short-term exposures (1 h to 4 d). A series of experiments are also presented that suggest a possible mechanism of the allelopathic effects observed.

MATERIALS AND METHODS

Cultures and culturing conditions. Three clones of *Cochlodinium polykrikoides*, CP1, CPPB12, and CPPB17, were examined for their allelopathic effects on other microalgae. The strains were obtained by pipetting single cells from seawater samples collected during blooms on August 31, 2006 from the most western basin of the Peconic Estuary, Flanders Bay (40.923° N, 72.587° W; for CP1) and September 4, 2008 from Peconic Bay (40.9368° N, 72.5128° W; for CPPB12 and CPPB17). The isolated single cells were transferred to polystyrene cell culture plates containing sterile GSe culture medium (see below) under an inverted microscope. Identification of all clonal isolates as *C. polykrikoides* has been confirmed with large subunit (LSU) rDNA sequencing (Gobler et al. 2008, Iwataki et al. 2008). Cells were cultured in sterile GSe medium with a salinity of 32.5, made with autoclaved and 0.2 µm filtered seawater (Doblin et al. 1999). The cultures were maintained at 21°C in an incubator with a 12 h light:12 h dark cycle, illuminated by a bank of fluorescent lights that provided a light intensity of ~100 µmol quanta m^{-2} s^{-1} to cultures.

The target species (strains) used in the present study included 5 species of dinoflagellates (*Akashiwo sanguinea* AS2, *Gymnodinium aureolum* KA2, *Gymnodinium instriatum* L6, *Heterocapsa rotundata* MS5, and *Scrippsiella* cf. *trochoidea* MS3), a diatom (*Thalassiosira weissflogii* CCMP1339), a pelagophyte (*Aureococcus anophagefferens* CCMP1984), a raphidophyte (*Chattonella marina* Chatt1), a haptophyte (*Isochrysis galbana*, Tahitian strain; T-Iso), and a cryptophyte (*Rhodomonas salina* CCMP1319). All CCMP strains were from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Maine, USA), while the *Chattonella marina* (ChatM1) from Singapore coastal waters was kindly provided by M. J. Holmes from the National University of Singapore and *I. galbana* (T-Iso) was kindly provided by Nichols S. Fisher's laboratory at Stony Brook University. All the other cultures were isolated by Y. Z. Tang from Chesapeake Bay (Virginia, USA; AS2, KA2, L6) or from Shinnecock Bay (New York, USA; MS3 and MS5). All the cultures were maintained under the same conditions as for *C. polykrikoides* cultures.

Since *Akashiwo sanguinea* was highly sensitive to *Cochlodinium polykrikoides* in preliminary experiments and easily identified in the co-culture with *C. polykrikoides*, it was used as a model target alga in multiple experiments. In addition, *A. sanguinea* AS2 and *G. instriatum* L6 were used for bi-algal culture experiments with *C. polykrikoides*, because these species are commonly found co-occurring with *C. polykrikoides* in the estuaries of the region (Gobler et al. 2007). All cultures used for experiments were in early or mid-exponential phase growth, with high levels of ambient nutrients still present. Additions of GSe medium were made to all experiments, and measured nitrate concentrations were >100 µM during all time points of all experiments. Given that these concentrations are 2 orders of magnitude above the half-saturation constant of nitrate for growth of *C. polykrikoides* (Gobler et al. unpubl. data), all experiments were performed under nutrient-replete conditions. In addition, pH levels always ranged between 7.8 and 9.0, in both treatments and control cultures, which were equivalent to or even smaller than the range of changes in pH in the cultures of *C. polykrikoides* and the target species, indicating treatment effects were not associated with changes in pH.

Generality of the allelopathic effects of *Cochlodinium polykrikoides*. To examine whether *C. polykrikoides* (strain CP1) had allelopathic effects on the co-occurring microalgal species and whether these effects were observable in particular species, 10 species of microalgae from different classes were chosen as target organisms to be co-cultured with *C. polykrikoides* CP1 (final cell density: 2450 ± 390 cells ml^{-1},

triplicates cultures) in 6-well culture plates for 24 h under the same conditions used for maintaining cultures. The initial cell biomass of all the target species was biovolume-normalized via dilution with GSe medium. Controls using the identical target cell densities and culture volumes were diluted with GSe medium rather than *C. polykrikoides*. Initial cell densities (i.e. after being combined with CP1 culture or, for controls only, with GSe medium) in treatments and controls of *Aureococcus anophagefferens*, *Rhodomonas salina*, *Isochrysis galbana*, *Thalassiosira weissflogii*, *Akashiwo sanguinea*, *Gymnodinium aureolum*, *Gymnodinium instriatum*, *Heterocapsa rotundata*, *Scrippsiella* cf. *trochoidea*, and *Chattonella marina* were 3.88 (±0.18) × 10^4, 2.9 (±0.27) × 10^4, 1.46 (±0.08) × 10^4, 3.66 (±0.01) × 10^3, 340 ± 60, 1.18 (±0.15) × 10^3, 250 ± 35, 1.24 (±0.58) × 10^3, 2.59 (±0.20) × 10^3, and 787 ± 189 cells ml^{-1}, respectively. During and at the end of the inoculation, the plates were observed and photographed with a digital SPOT Insight camera mounted on a Nikon Eclipse TS100 inverted microscope (Nikon) to document possible morphological and behavior changes and cell death at 4 and 24 h. The triplicate cultures were fixed with Lugol's solution (final concentration: 2%), and cell densities were enumerated with a 0.1 ml phytoplankton counting chamber or a 1.0 ml Sedgewick rafter counting chamber under a compound microscope (each sample of triplicates for a treatment was counted thrice).

Allelopathic effects of multiple strains of *Cochlodinium polykrikoides*. To determine whether the allelopathic effects observed from *C. polykrikoides* were a feature specific to strain CP1, experiments were conducted with 2 additional strains of *C. polykrikoides*: CPPB-12 (targeting *Gymnodinium instriatum* L6) and CPPB-17 (targeting *Akashiwo sanguinea*), while strain CP1 was used for positive controls. Using 12-well culture plates, cultures of CP1, CPPB-12 and CPPB-17 were added to triplicate wells along with cultures of *A. sanguinea* or *G. instriatum*. Control treatments for each species and strain were also established with GSe medium added in place of the co-culture. The initial cell densities of CP1, CPPB-17, and AS2 were 730, 870, and 40 cells ml^{-1}, respectively, for the first experiment, while initial cell densities of CP1, CPPB-12, and L6 were 185, 155, and 80 cells ml^{-1}, respectively, for the second. Culture plates were incubated using the conditions listed above for 24 h, after which cultures were preserved with Lugol's solution (final concentration: 2%) for enumeration.

Dose-dependence of allelopathic effects of *Cochlodinium polykrikoides*. Experiments examining how the allelopathic effects changed with the cell densities of *C. polykrikoides* and target species were conducted. Two bi-algal culture experiments were conducted using

triplicate 200 ml Pyrex Erlenmeyer flasks, each containing either *C. polykrikoides* CP1 (control), a target species (*Akashiwo sanguinea*, *Gymnodinium instriatum*; control), or a mixture of *C. polykrikoides* CP1 and a target species (treatment). All flasks were incubated under the same conditions used for maintaining cultures, and aliquots of cultures (5 ml each) were preserved with Lugol's solution at time points of 24 h or up to 48–168 h at intervals of 24 h and were enumerated as above. The first experiment was conducted with varying cell densities of *C. polykrikoides* CP1 and *A. sanguinea*: 1000 cells ml^{-1} *C. polykrikoides* CP1 with 1000, 590, 290, 210, and 130 cells ml^{-1} *A. sanguinea*, or 1000 cells ml^{-1} *A. sanguinea* with 570, 330, 230, and 110 cells ml^{-1} CP1. The second experiment was conducted with 750 cells ml^{-1} *C. polykrikoides* (with 640, 380, 130, and 80 cells ml^{-1} *G. instriatum*) and with 370 cells ml^{-1} *C. polykrikoides* (with 720 cells ml^{-1} *G. instriatum*).

Allelopathic effects of different treatments and fractions of *Cochlodinium polykrikoides*. To better understand the nature of the allelopathic effects of *C. polykrikoides* on other microalgae, experiments were conducted in which the physiological status of *C. polykrikoides* or the chemical features of *C. polykrikoides* cultures were manipulated. Specifically, the following treatments of *C. polykrikoides* CP1 cultures (cell density: 1.15×10^3 cells ml^{-1}) were used: heating (100°C for 15 min) and cooling (to 21°C); freezing (–80°C for 30 min) and thawing at 21°C; removal of cells by filtration through a 5 µm mesh nylon membrane; sonication of cells with a high power sonicator (Ultrasonic Power Corporation; the intactness of cells was checked visually under microscope); and addition of the ROS-scavenging enzymes peroxidase (2.5 µg ml^{-1}) and catalase (1.0 U ml^{-1}) to the culture immediately before the experiment (Tang & Gobler 2009a). *Akashiwo sanguinea* at a final cell density of 450 cells ml^{-1} was co-cultured with CP1 after these treatments (final CP1 cell density or equivalent: 920 cells ml^{-1}) or with untreated *C. polykrikoides* CP1 cultures (final cell density: 920 cells ml^{-1}; positive control) in triplicate 200 ml Erlenmeyer flasks. A monoculture of *A. sanguinea* at the same final cell density as above was used as a negative control. All flasks were incubated for 24 h under the same conditions used for culture maintenance, subsequently cultures were preserved with Lugol's solution (final concentration: 2%) and cell densities for both *A. sanguinea* and CP1 were enumerated using a Sedgewick rafter counting chamber.

Allelopathic effects of cell-free medium connected to a live culture of *Cochlodinium polykrikoides*. To explore whether direct contact between *C. polykrikoides* cells and target cells is needed for *C. polykrikoides* to impart allelopathic effects, experiments were conducted exposing target cells to the cell-free medium that was connected to an active culture of *C. polykrikoides* CP1 via a 5 µm mesh nylon barrier (Sefar Filtration Inc.). The experiments were performed in a specifically modified plastic (high density polyethylene, HDPE) chamber with 30 compartments ($4 \times 4 \times 4$ cm), in which pairs of 2 adjacent compartments were separated with 5 µm mesh nylon (Tang & Gobler 2009a). A snap-shut lid for the chamber allowed for UV-sterilization and easy access to all individual compartments at the start and end of all experiments.

Three experiments were conducted with the target species *Akashiwo sanguinea* (exposure time 72 h), *Gymnodinium instriatum* (exposure time 72 h), and *Chattonella marina* (exposure time 144 h). For each experiment, each chamber contained 30 ml of either one of the following 4 cultures in triplicate: CP1 control (in both sides of the 2 membrane-partitioned chambers), the target species control (in both sides of the 2 membrane-partitioned chambers), a bi-algal mixture of CP1 and a target species with cell densities the same as controls (positive control), and the culture of a target species connected to the culture of CP1 through the 5 µm membrane. The initial concentrations of *A. sanguinea*, *G. instriatum*, and *C. marina* in the bi-algal mixtures were 640, 600, and 500 cells ml^{-1}, respectively, while the corresponding initial cell concentrations of *Cochlodinium polykrikoides* CP1 were 1740, 1650, and 900 cells ml^{-1}, respectively. After the cultures were added, the chamber was closed and incubated under the standard conditions listed above. After incubation, the cultures were gently mixed, and pipetted simultaneously from both sides by 2 people, ensuring that the rate of culture removal was identical for each side. Samples were fixed with Lugol's solution (final concentration: 2%) and microscopically enumerated using a Sedgewick rafter counting chamber.

Natural algal population experiments. Two experiments were conducted with *Cochlodinium polykrikoides* bloom water or non-bloom water to observe the allelopathic effects of *C. polykrikoides* cultures and blooms within a natural phytoplankton community. For the experiment with bloom water, a surface bloom (with a *C. polykrikoides* cell density of 2700 cells ml^{-1}) was sampled from Peconic Bay, Long Island, New York, USA, on September 2, 2009. In a 200 ml sterile flask, 10 ml of *Akashiwo sanguinea* culture (final concentration: 10 cells ml^{-1}) was mixed with 40 ml of bloom water as a treatment. Two more aliquots of *A. sanguinea* culture were mixed with either GSe medium or 0.2 µm filtered (Steriflip; Millipore) bloom water as controls. After gentle through-mixing, triplicate 10 ml aliquots were transferred into 12 ml wells of 6-well plates (Corning) and incubated under standard conditions for 24 h. Triplicate 10 ml aliquots were also

preserved with Lugol's solution (final concentration: 2%) at the start of experiments and after 24 h incubation for enumeration of cell concentrations. The salinities of bloom water and *A. sanguinea* culture were 29 and 32, respectively.

For the experiment with non-bloom water, surface water was sampled on August 22, 2009 from Old Fort Pond (OFP), a tidal tributary of Shinnecock Bay, Long Island. In triplicate 200 ml sterile flasks, a 50 ml aliquot of a *Cochlodinium polykrikoides* CP1 culture (final cell density: 2100 cells ml^{-1}) was combined with 50 ml seawater from OFP as the treatment. The *C. polykrikoides* culture and natural community were both mixed with 50 ml of 0.2 µm mesh filtrated OFP seawater as 2 distinct controls. After gentle mixing, triplicate 10 ml aliquots of each treatment were transferred into 12 ml wells of 6-well plates, which were then incubated under standard conditions for 24 h. Initial and 24 h samples were preserved with Lugol's solution for enumeration of the 6 most abundant microphytoplankton: the diatoms *Skeletonema* cf. *costatum*, *Chaetoceros* sp. and *Thalasiossira* sp., the flagellate *Euglena* sp., and the dinoflagellates *Gyrodinium* sp. and *Scrippsiella* sp. The salinities of seawater and culture were 30 and 32, respectively, measured before the experiment.

Statistics. One-way ANOVAs and *t*-test were performed to assess the effects of different treatments of *Cochlodinium polykrikoides* cultures (e.g. concentrations, direct or indirect exposure, live or killed cultures, addition of enzymes) and/or the duration of exposure to *C. polykrikoides* cells. All percentage data were arcsine square root–transformed before performing ANOVA. Differences among treatments were generally assessed with Holm-Sidak post hoc pairwise comparisons using SigmaStat 3.1. In all cases, significance levels were set at $p < 0.05$.

RESULTS

Allelopathic effects on competing species

Cochlodinium polykrikoides CP1 exhibited strong allelopathic effects on all target species examined (Figs. 1 & 2). The specific effects include loss of flagella and thus motility (for flagellates *Akashiwo sanguinea*, *Gymnodinium aureolum*, *Gymnodinium instriatum*, *Heterocapsa rotundata*, *Scrippsiella* cf. *trochoidea*, and *Chattonella marina*), change of cellular morphology into a round shape (*Rhodomonas salina*,
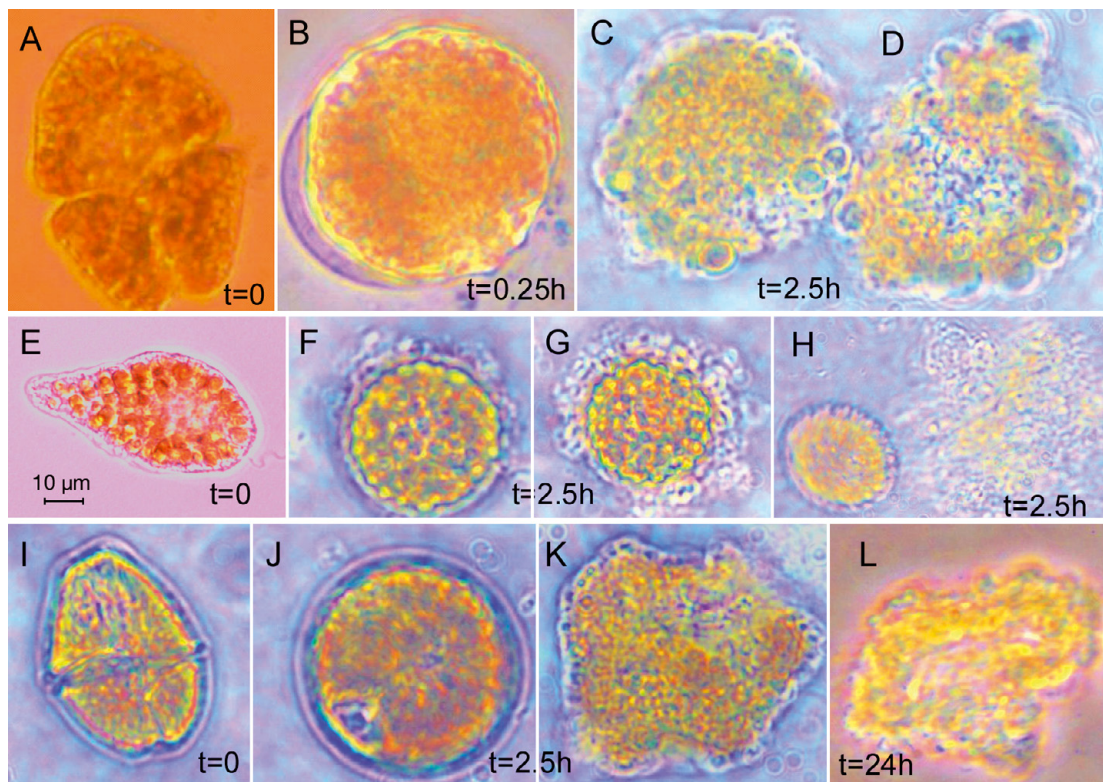

Fig. 1. (A to D) *Akashiwo sanguinea*, (E to H) *Chattonella marina*, and (I to L) *Gymnodinium instriatum*. Morphology of selected target microalgae during exposure to *Cochlodinium polykrikoides* CP1: (A,E,I) normal cells, (B,F,J) cells that have lost flagella and motility and changed shape in general, (C,G,K) substantially deformed cells, and (D,H,L) lysed cells. Exposure time (t) is as shown
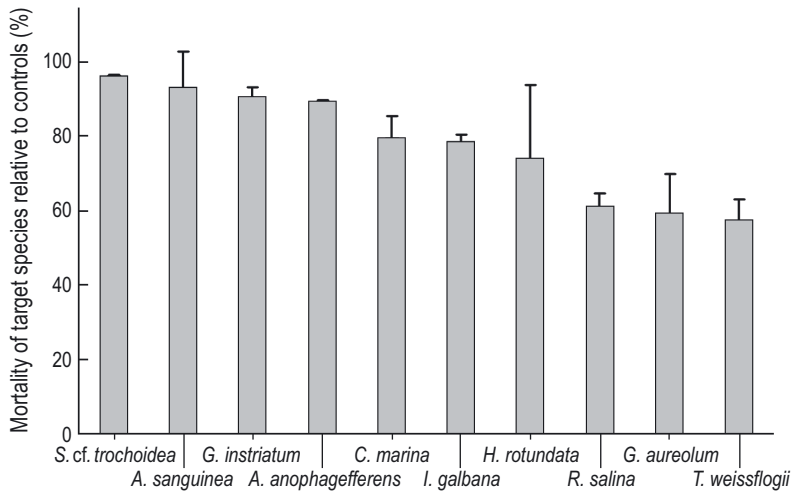
Fig. 2. Percent cell mortality of 10 target species (*Scrippsiella* cf. *trochoidea*, *Akashiwo sanguinea*, *Gymnodinium instriatum*, *Aureococcus anophageffer-ens*, *Chattonella marina*, *Isochrysis galbana*, *Heterocapsa rotundata*, *Rhodo-monas salina*, *Gymnodinium aureolum*, *Thalassiosira weissflogii*) co-cultured with *Cochlodinium polykrikoides* CP1 (2450 ± 390 cells ml^{-1}) for 24 h, expressed as the percent mortalities of the target cells relative to their respective controls. Error bars: +1 standard deviation of n = 3

A. sanguinea, *G. aureolum*, *G. instriatum*, *H. rotun-data*, *S.* cf. *trochoidea*, and *C. marina*), and eventual cell disintegration and lysis (Fig. 1). These effects were observed in 15 min in some *A. sanguinea* cells, but took hours in other species (Fig. 1). After 24 h, *C. polykrikoides* CP1 at densities of 2450 cells ml^{-1} caused mortalities in *Aureococcus anophagefferens*, *R. salina*, *Isochrysis galbana*, *Thalassiosira weissflogii*, *A. sanguinea*, *G. aureolum*, *G. instriatum*, *H. rotun-data*, *S.* cf. *trochoidea*, and *C. marina* of 90, 61, 79, 57, 93, 59, 91, 74, 96, and 79% compared to control cultures, respectively (p < 0.001; Fig. 2). These results demonstrated that the allelopathic effects of *C. polykrikoides* were not specific to any species or class of algae, although some species (*A. anophagefferens*, *A. sanguinea*, *G. instriatum*, *S.* cf. *trochoidea*) exhibited greater sensitivity (≥90% mortality) than others (*R. salina*, *I. galbana*, *T. weissflogii*, *G. aureolum*, *H. rotundata*, *C. marina*; 57 to 79%; Fig. 2).

Allelopathic effects of different strains

In experiments using different strains of *Cochlo-dinium polykrikoides* (CP1, CPPB-12, and CPPB17) and the target species *Akashiwo sanguinea* and *Gymnodinium instriatum*, both strains CP1 (730 cells ml^{-1}) and CPPB-17 (870 cells ml^{-1}) caused 100% cell mortality in *A. sanguinea* in 24 h (Fig. 3A), while both strains CP1 (185 cells ml^{-1}) and CPPB-12 (155 cells ml^{-1}) caused significantly higher cell mortality in *G. instriatum* compared to control cultures after 24 h

(67 and 35%, respectively; p < 0.001, *t*-test; Fig. 3B). These results demonstrated that the allelopathic effects exhibited in *C. poly-krikoides* are not strain-specific among strains isolated from different years and locations in New York.

Effects of cell densities and exposure time

In bi-algal cultures of *Cochlodinium poly-krikoides* (110 to 1000 cells ml^{-1}) and *Akashiwo sanguinea* (1000 cells ml^{-1}), the cell mortality of *A. sanguinea* increased dramatically (ANOVA, p < 0.001) with increasing density of *C. polykrikoides* (Fig. 4A). For example, *C. polykrikoides* at 330 cells ml^{-1} caused mortality of ~40% of *A. sanguinea* cells after 24 h, while 1000 cells ml^{-1} caused 80% mortality (Fig. 4A). In bi-algal cultures with *C. polykrikoides* densities fixed at 1000 cells ml^{-1}, the mortality of *A. sanguinea* increased significantly (ANOVA, p < 0.001) with decreasing *A. sanguinea* cell density, reaching 100% when the cell density of *A. sanguinea* was ≤290 cells ml^{-1} (Fig. 4B). Hence, these allelopathic ef-
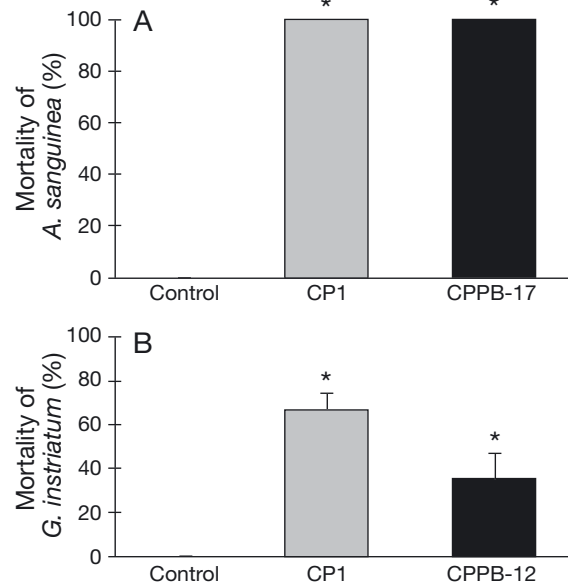

Fig. 3. (A) Percent cell mortality of *Akashiwo sanguinea* during 24 h co-culture with *Cochlodinium polykrikoides* strains CP1 and CPPB-17 relative to the *A. sanguinea* control. The average initial cell concentrations of CP1, CPPB17, and *A. sanguinea* were 730, 870, and 40 cells ml^{-1}. (B) Percent cell mortality of *Gymnodinium instriatum* in 24 h co-cultured with *C. polykrikoides* strains CP1 and CPPB-12 relative to the *G. instriatum* control. The average initial cell concentrations of CP1, CP12, and *G. instriatum* were 185, 155, and 80 cells ml^{-1}. Asterisks: significant differences (p < 0.05) between treatments and control; error bars: +1 standard deviation of n = 3
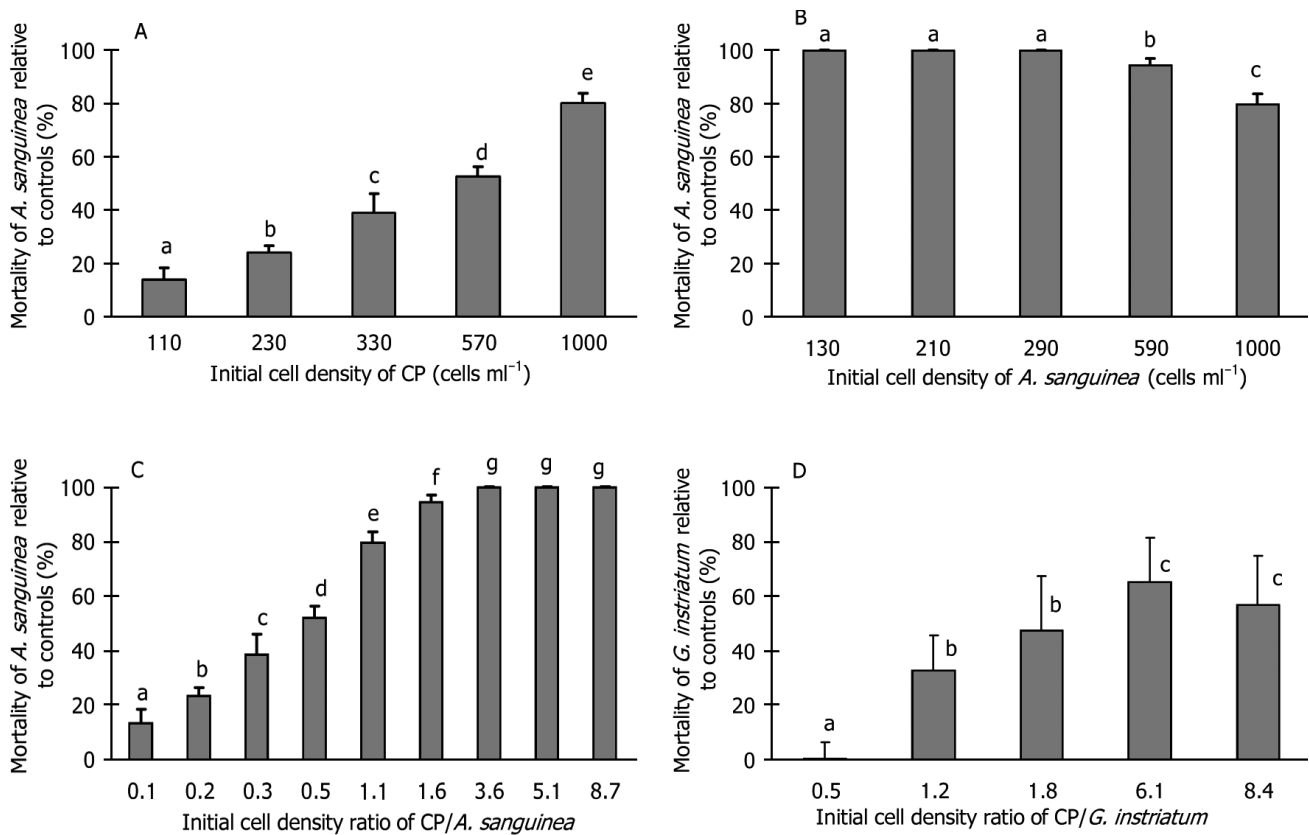
Fig. 4. Percent cell mortality of the target species (*Akashiwo sanguinea*, *Gymnodinium instriatum*) compared to the respective controls as a function of the initial cell densities of *Cochlodinium polykrikoides* (strain CP1), *A. sanguinea*, and the ratio of *C. polykrikoides* to the target species in bi-algal cultures. (A) *A. sanguinea* was fixed (1000 cells ml^{-1}) and *C. polykrikoides* varied (110 to 1000 cells ml^{-1}) over 24 h; (B) *C. polykrikoides* was fixed (1000 cells ml^{-1}) and *A. sanguinea* varied (130 to 1000 cells ml^{-1}) over 24 h; (C) *C. polykrikoides* and *A. sanguinea*, with initial cell densities that varied (1000 cells ml^{-1} *C. polykrikoides* vs. 130 to 1000 cells ml^{-1} *A. sanguinea* and 1000 cells ml^{-1} *A. sanguinea* vs. 110 to 570 cells ml^{-1} *C. polykrikoides*) and 24 h culturing; (D) *C. polykrikoides* and *G. instriatum*, with initial cell densities that varied (750 cells ml^{-1} *C. polykrikoides* vs. 640, 380, 130, and 80 cells ml^{-1} *G. instriatum* and 370 cells ml^{-1} *C. polykrikoides* vs. 720 cells ml^{-1} *G. instriatum*) and 24 h culturing. Each data point was calculated from the mean of treatments (n = 3) and their corresponding controls (n = 3). Error bars: +1 standard deviation of n = 3; different lower case letters indicate significant differences (p < 0.05) among treatments

fects depended on the absolute cell density of both *C. polykrikoides* and *A. sanguinea* (Fig. 4A, B), with the percent cell mortality of *A. sanguinea* increasing hyperbolically with the ratio of *C. polykrikoides*:*A. sanguinea* cells (Fig. 4C). *A. sanguinea* displayed nearly 100% mortality when the ratio of *C. polykrikoides*:*A. sanguinea* exceeded 1.0 (ANOVA, p < 0.001; Fig. 4C). A second experiment conducted with *C. polykrikoides* and the target species *G. instriatum* demonstrated a similar dependence of the allelopathic effects on the initial ratio of *C. polykrikoides*:*G. instriatum* (Fig. 4D). At *C. polykrikoides*:*A. sanguinea* ratios exceeding 2, there was >50% mortality in *G. instriatum*, whereas at ratios of 1.2 and 0.5, compared to the control, 33 and 0%, respectively, of *G. instriatum* cells were lost (Fig. 4C).

The allelopathic effects of *Cochlodinium polykrikoides* also depended on exposure time. With initial concentrations of *C. polykrikoides* and *Akashiwo sanguinea* at 800 and 500 cells ml^{-1}, respectively, cell mortality in *A. sanguinea*, which was calculated in comparison with the control, significantly increased from 60 to 95% with an increase in exposure time from 1 to 7 d (ANOVA, p < 0.001; Fig. 5). There was no significant change in *C. polykrikoides* cell densities over this time frame.

Effects of direct/indirect cell contact

Cultures of *Cochlodinium polykrikoides* CP1 separated from target algae with 5 µm mesh nylon membranes exhibited significant allelopathic effects on *Akashiwo sanguinea*, *Gymnodinium instriatum*, and *Chattonella marina* (ANOVA, p < 0.001; Fig. 6). Dur-
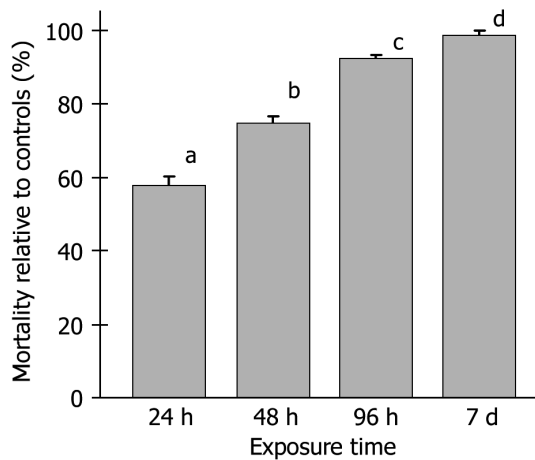
Fig. 5. Percent cell mortality of the target species *Akashiwo sanguinea* relative to the respective controls (%) as a function of the exposure (co-culturing) time of *A. sanguinea* to *Cochlodinium polykrikoides* CP1. The initial cell densities of CP1 and *A. sanguinea* were 800 and 500 cells ml^{-1}, respectively. Each data point was calculated from the mean of treatments (n = 3) and their corresponding controls (n = 3). Error bars: +1 standard deviation of n = 3; different lower case letters indicate significant (p < 0.05) differences among treatments

ing a 72 h exposure of 640 cell ml^{-1} of *A. sanguinea* to 1740 cells ml^{-1} of CP1, cell densities of *A. sanguinea* declined to 130 cells ml^{-1}, while indirect exposure (5 µm mesh nylon; Fig. 6) led to a decrease to 540 cells ml^{-1}, which is significantly higher than with

the direct exposure (ANOVA Holm-Sidak post hoc pairwise comparison, p <0.001), but significantly lower than the negative control (710 cells ml^{-1}; ANOVA Holm-Sidak post hoc pairwise comparison, p < 0.001). Direct exposure of *C. marina* (475 cells ml^{-1}) to CP1 (1780 cells ml^{-1}) led to a decrease in the concentration of *C. marina* to 206 cells ml^{-1}, while the indirect exposure led to growth of *C. marina* to 2400 cells ml^{-1}, which was significantly less than that of the negative control of *C. marina* (3360 cells ml^{-1}; ANOVA Holm-Sidak post hoc pairwise comparison, p < 0.001; Fig. 6). Similarly, direct exposure of *G. instriatum* (600 cells ml^{-1}) to CP1 (1420 cells ml^{-1}) for 72 h led to a slight increase of *G. instriatum* to 780 cells ml^{-1}, while the indirect exposure led to growth of *G. instriatum* to 1280 cells ml^{-1}, which was significantly higher than that of the direct exposure, but significantly less than that of the negative control of *G. instriatum* (1570 cells ml^{-1}; ANOVA Holm-Sidak post hoc pairwise comparison, p <0.001; Fig. 6). All the above results indicated that direct contact between *C. polykrikoides* cells and the cells of target species is not necessary for *C. polykrikoides* to have an allelopathic effect and that at least some of the allelochemicals responsible are released from cells of *C. polykrikoides*, although direct exposure of target species to *C. polykrikoides* caused a much stronger effect on target species.
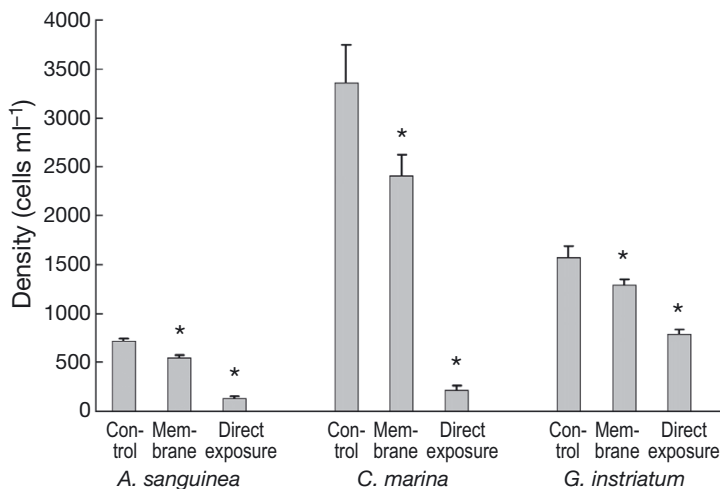

Fig. 6. Cell densities of *Akashiwo sanguinea*, *Chattonella marina*, and *Gymnodinium instriatum* following co-culturing with direct and indirect exposure to *Cochlodinium polykrikoides* CP1 via a 5 µm nylon mesh for 72 h. Concentrations of each target species (*A. sanguinea*, *C. marina*, and *G. instriatum*) were constant in the negative control (target species only, the indirect treatment (membrane partitioned), and the direct exposure (mixed culture). Concentrations of *C. polykrikoides* in the treatment chamber and in the mixed culture were the same for each test species. Error bars: +1 standard deviation of n = 3; asterisks: significant difference (p < 0.05) from the control and between the direct and indirect exposures

Effects of pre-treatment of *Cochlodinium polykrikoides* and addition of enzymes

Cochlodinium polykrikoides CP1 cultures at 920 cells ml^{-1} completely lost their toxicity or allelopathic effects on *Akashiwo sanguinea* (with an initial cell density of 450 cells ml^{-1}) zsonication, and filtration (cell-free culture medium). Final cell densities of *A. sanguinea* in each of these treatments were not different from the negative control (without *C. polykrikoides*) (ANOVA post hoc comparison, p > 0.05; Fig. 7). In contrast, 98% *A. sanguinea* cells in the positive control culture lysed after 24 h exposure to untreated *C. polykrikoides* cells (Fig. 7). The addition of 2.5 µg ml^{-1} of peroxidase (final concentration) to the experimental cultures mitigated this impact, as there was no significant difference in the final cell density of *A. sanguinea* between this treatment and the negative control cultures (ANOVA post hoc comparison, p > 0.05; Fig. 7). The addition of catalase (1.0 U ml^{-1}) yielded a final cell density of *A. sanguinea* that was significantly higher than the positive control (ANOVA post
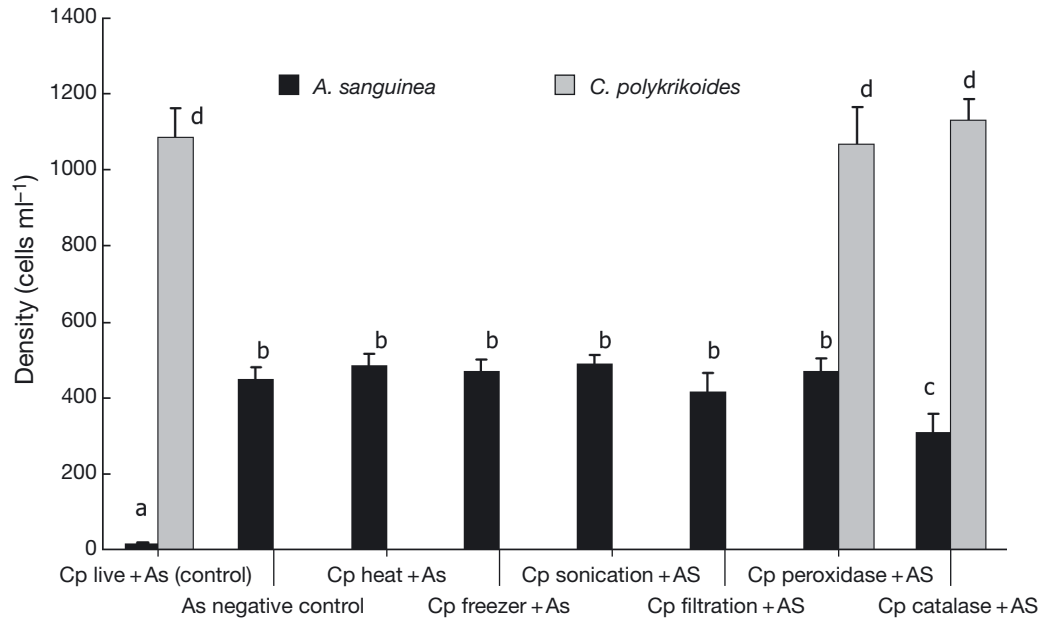
Fig. 7. Cell densities of *Akashiwo sanguinea* (As) and *Cochlodinium polykrikoides* CP1 (Cp) after heating (100°C for 15 min), freezing (–80°C), sonication, filtration (through 5 µm mesh membrane), and with additions of peroxidase (final concentration: 2.5 µg ml^{-1}) or catalase (final concentration: 1.0 U ml^{-1}). Error bars: +1 standard deviation of n = 3; different lowercase letters: significant differences (p < 0.05)

hoc comparison, p < 0.001; Fig. 7), but significantly lower than the negative control (ANOVA post hoc comparison, p < 0.001; Fig. 7), i.e. addition of catalase removed most, but not all, of the allelopathic effects of *C. polykrikoides* on *A. sanguinea*. There was no significant difference in cell density of *C. polykrikoides* CP1 among the positive control and the mixed cultures with addition of peroxidase or catalase (Fig. 7). *A. sanguinea* cultures grown with and without these enzymes at these concentrations yielded nearly identical cell densities (ANOVA post hoc comparison, p > 0.05).

Allelopathic effects of *Cochlodinium polykrikoides* on natural phytoplankton communities

Akashiwo sanguinea cells added to a natural sample collected during a bloom of *Cochlodinium polykrikoides* from the Peconic Estuary experienced significant mortality (76%), *A. sanguinea* did not grow in the bloom water filtrate control, but doubled in cell density in the GSe medium control (ANOVA post hoc pairwise comparison, p < 0.001; Fig. 8). During 24 h exposure of a natural phytoplankton community to a culture of *C. polykrikoides* CP1 (2100 cells ml^{-1}), 5 of the 6 most abundant phytoplankton species (*Chaetoceros* sp., *Gyrodinium* sp., *Scrippsiella* sp., *Skeletonema costatum*, and *Thalasiossira* sp.) exhibited significantly lower cell densities compared to the control

treatment (p < 0.001, *t*-test; Fig. 9). The single exception to this trend was *Euglena* sp., which achieved higher cell densities in the presence of the *C. polykrikoides* culture (p < 0.001, *t*-test; Fig. 9).
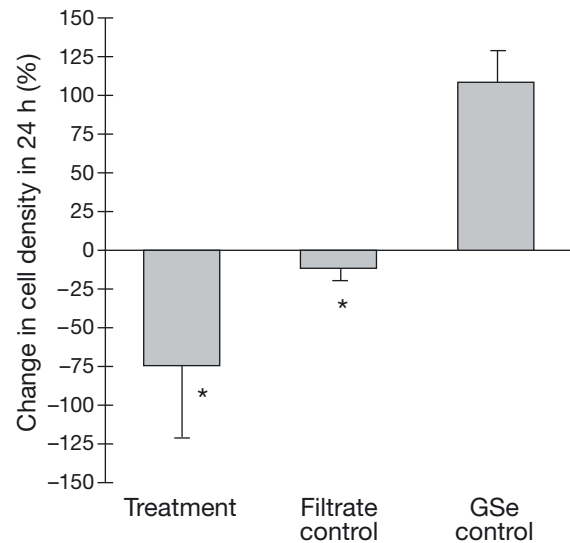

Fig. 8. Percent change in cell densities of *Akashiwo sanguinea* co-cultured with *Cochlodinium polykrikoides* bloom water from the Peconic Estuary for 24 h. Cultures of *A. sanguinea* cultured in either GSe medium or 0.2 µm mesh filtrated bloom water were used as controls. Asterisks: significant differences (p < 0.05) between treatment and controls or between 2 controls in percent change in cell densities in 24 h incubation; error bars: ±1 standard deviation of n = 3
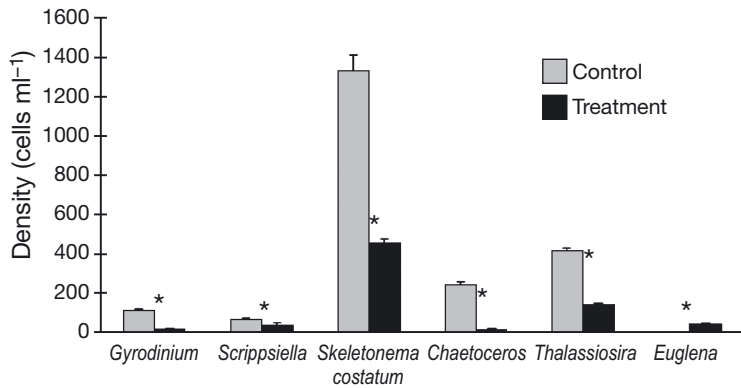
Fig. 9. Cell densities of dominant species present in the water from Old Fort Pond, co-cultured with *Cochlodinium polykrikoides* CP1 and with 0.2 µm mesh filtered *C. polykrikoides* culture medium after 24 h incubation. Asterisks: significant differences (p < 0.05) between treatment and control in cell density for each species; error bars: +1 standard deviation of n = 3

DISCUSSION

Possible mechanisms and chemical nature of the allelopathic effects

The present study demonstrates that clonal cultures and blooms of *Cochlodinium polykrikoides* from the coast of the northeastern United States have highly toxic and inhibitory effects on a variety of common phytoplankton species from multiple classes. The observations suggest the allelopathic effects of *C. polykrikoides* are broad and not specific to any particular taxon of microalgae. The allelopathic effects on some species were observable within minutes, and included loss of motility, change of cell morphology, and cell lysis and cell death occurred within hours, characteristics categorized as 'acute toxicity' according to Tillmann et al. (2008b). The allelopathic effects depended on the absolute and relative cell abundance of *C. polykrikoides* (i.e. high absolute and relative abundances of *C. polykrikoides* yielded higher mortalities in target phytoplankton) and the exposure time (i.e. longer co-culturing caused higher mortality in the cells of target species). Treatments of heating, freezing, sonication, and filtration for the culture of *C. polykrikoides* led to significant or complete loss of its allelopathic effects on *Akashiwo sanguinea*, suggesting that the allelopathic potency depends on viability (or physiological activity) of *C. polykrikoides* cells, and the agents responsible were highly short-lived. The allelopathic effects exhibited by *C. polykrikoides* cultures connected with the culture of target species (*A. sanguinea*, *Chattonella marina*, or *Gymnodinium instriatum*) through a 5 µm mesh membrane indicated that *C. polykrikoides* produces allelopathic agents that could

be released and dissolved in culture medium. Therefore, although direct contact between *C. polykrikoides* cells and target cells maximizes allelopathic effects, direct contact is not a requisite condition for *C. polykrikoides* to exhibit allelopathy. Yamasaki et al. (2007) reported that direct cell contact between *C. polykrikoides* and *A. sanguinea* was required for the growth-inhibitory and morphological effects observed in *A. sanguinea* cells and thus excluded allelopathy as a mechanism. Yamasaki et al. (2007) reported the absence of an allelopathic effect in the cell-free medium that was connected with an active *C. polykrikoides* culture (100 cells ml^{-1}) through a 3.0 µm mesh membrane. We have found that this density of *C. polykrikoides* is too low to elicit an allelopathic effect in other species (data not shown). Additionally, the smaller and different membrane may not have permitted *C. polykrikoides* allelochemicals to diffuse in the same manner as our 5 µm nylon mesh did.

The removal of allelopathic effects by addition of the ROS-scavenging enzymes peroxidase and catalase into *Cochlodinium polykrikoides* cultures implies that ROS-like chemicals (e.g. O_2^-, OH·, HO_2·, ROO^-) are responsible for the alga-killing effect of *C. polykrikoides*. The loss of toxicity of *C. polykrikoides* following heating, freezing, sonication, and filtration, and the reduced, but still measurable, toxicity of cultures partitioned with nylon mesh membrane collectively indicate that allelopathic chemicals are actively produced by live cultures of this species and have a maximal impact when contact or close proximity exists between live cells of *C. polykrikoides* and the target algae. These results are all consistent with our previous investigations of the toxic effects of *C. polykrikoides* on fish and shellfish larvae using the same cultures of *C. polykrikoides* and treatments (Tang & Gobler 2009a,b). This consistency strongly suggests that, unlike other harmful algae whose well-known animal-killing toxins and allelochemicals are not identical (e.g. the paralytic shellfish toxin-producer *Alexandrium* spp.: Tillmann & John 2002, Tillmann et al. 2007, 2008a; the brevetoxins-producer *Karenia brevis*: Kubanek et al. 2005), agents produced by *C. polykrikoides* which are toxic to fish, shellfish, and shellfish larvae are also responsible for the allelopathic effects to other phytoplankton. Therefore, *C. polykrikoides* may provide another case to the short list of HAB species that have been proven to have a common mechanism for their toxicity to animals and allelopathic effects on phytoplankton (*Karlodinium veneficum* and *Prymnesium parvum*: Granéli & Hansen 2006, Tillmann et al. 2008b). The consistency between allelopathic experiments, fish bioassays,

bivalve bioassays, and bioassays of shellfish larvae and the non-specificity of allelopathic effects on all micro-algae tested suggests that labile, ROS-like chemicals are the allelopathic agents in *C. polykrikoides*, although further studies are needed to confirm this hypothesis.

It has been previously suggested that elevated pH values in mixed cultures of donor and target species might be responsible for the observed allelopathic-like effects in some HABs (Schmidt & Hansen 2001, Lundholm et al. 2005). However, the lack of significant differences among pH levels in cultures of *Cochlodinium polykrikoides*, target species (i.e. no difference), and mixed cultures in the present study demonstrated that pH shock or pH increase during co-culturing was not responsible for the allelopathic effects of *C. polykrikoides*. Moreover, the presences of nitrate at levels more than an order of magnitude above the saturation constant for growth of *C. polykrikoides* (Gobler et al. unpubl. data) suggests that nutrient limitation would not account for the dramatic and prompt results we obtained (e.g. >90% cell lysis in target species in ≤24 h; Fig. 2).

Ecological implications of the allelopathic effects

The production of allelopathic chemicals is a strategy used by harmful algae to outcompete other, co-existing phytoplankton (Smayda 1997, Granéli & Hansen 2006, Tillmann et al. 2008b), and our results demonstrate that this strategy likely plays a role in the occurrence of *Cochlodinium polykrikoides* blooms. In cultures, *C. polykrikoides* is a slow-growing alga ($\mu = 0.4$ d^{-1}; Tang pers. obs.) compared to other diatoms and dinoflagellates, including *Gymnodinium instriatum* and *Akashiwo sanguinea* (Smayda 1997), which bloom in the estuaries of New York prior (1 to 4 wk) to *C. polykrikoides* blooms or coexist with *C. polykrikoides* at high densities during bloom initiation (Gobler et al. 2007, Tang & Gobler pers. obs.). Given this disadvantage in growth, *C. polykrikoides* must rely on other mechanisms, such as allelopathy to form mono-specific blooms. Since *C. polykrikoides* causes dramatic mortality in planktonic grazers, including bivalve larvae (Tang & Gobler 2009b), planktivorous fish (Tang & Gobler 2009a), and zooplankton (Jiang et al. 2009), grazer deterrence is another process that likely contributes to bloom formation. Allelopathic effects on competitors, together with grazing deterrence, could promote *C. polykrikoides* blooms through positive feedback (Sunda et al. 2006), whereby higher *C. polykrikoides* cell densities yield fewer competitors and predators, which, in turn, facilitates higher cell densities. Since the allelopathic effects of *C. polykrikoides* on co-occurring algae depend on absolute and relative cell abundances, allelo-pathy is more likely to contribute toward bloom maintenance when cell densities are high, than to bloom initiation when cell densities are low. The ability of *C. polykrikoides* to form localized areas of high cell densities by diurnal migration (Gobler et al. 2008, Kudela et al. 2008), however, could facilitate a scenario whereby *C. polykrikoides* could have allelopathic effects on competing algae, even during bloom initiation. In light of the conclusions of Putnam & Tang (1986), Smayda (1997), and Tillmann et al. (2008b), further confirmative studies identifying *C. polykrikoides* allelochemicals and/or toxins and *in situ* measurements of allelopathic agents and their bioactivity are needed to clarify the role of allelopathy in *C. polykrikoides* blooms, particularly during bloom initiation.

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